INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Université d'Ottawa · University of Ottawa

FAS AND FAS LIGAND EXPRESSION AND APOPTOSIS IN CISPLATIN-SENSITIVE AND -RESISTANT HUMAN OVARIAN EPITHELIAL CANCER CELLS

by

Danielle Schneiderman

© Thesis submitted to the School of Graduate Studies and Research, University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science, Department of Cellular and Molecular Medicine, Faculty of Medicine

National Library of Canada
Bibliothèque nationale du Canada

Acquisitions and Bibliographic Services
Acquisitions et services bibliographiques

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-48179-4

Canada

1.0 ACKNOWLEDGEMENTS

I wish to express my thanks to my supervisor Dr. Benjamin K. Tsang. His patience, enthusiasm, encouragement and scientific guidance have helped me stay motivated, learn a great deal and provided a stimulating working environment for my professional development.

I wish to thank Dr. Barabara Vanderhyden, Dr. Henry Fliss and Dr. Jackie Carnegie, members of my Advisory Committee. Their constructive criticism and input has helped in the progress of my project.

I wish to thank Dr. Jong Min Kim, whose patience, enthusiasm and scientific and technical guidance were invaluable in shaping my project over the past two years. I would like to acknowledge Dr. Kim's contribution of the immunohistochemistry of human ovarian tumor tissue. I would also like to thank Dr. Chawou Xiao for his guidance and technical support as well as his contribution to Figures 15 and 22. As well, I would like to thank Dr. David Boone for his technical expertise and advice, and Dr. Hiromasa Sasaki, Dr. Jonathan Soboloff and (soon to be Dr.) Karen Phillips for providing a stimulating work environment and for their support.

I wish to dedicate this thesis to my husband David Schneiderman. He provided constructive criticism and stimulating discussion throughout my project and of course his never ending support.

Last, but not least, I wish to thank the Government of Canada and particularly the Natural Science and Engineering Research Council of Canada for providing me with financial support in the form of a scholarship throughout my studies.

2.0 ABSTRACT

Cisplatin (CDDP) derivatives are first line chemotherapeutic agents for the treatment of ovarian epithelial cancer. The cytotoxic effect of these agents are believed to be mediated through the induction of apoptosis and defects in the apoptotic mechanism may contribute to chemoresistance. The role of the Fas/FasL system in mediating drug-induced apoptosis and its involvement in chemoresistance is not fully understood. In the present study, we have used cultures of established cell lines of CDDP-sensitive human ovarian epithelial tumours (OV2008 and A2780-s) and their resistant variants (C13* and A2780-cp, respectively) to assess the role of Fas/FasL system in the chemo-responsiveness of ovarian cancer cells to CDDP. CDDP was effective in inducing the expression of cell-associated Fas and FasL, sFasL and apoptosis in a concentration and time-dependent fashion in both OV2008 and A2780-s cell lines. In contrast, while CDDP was effective in increasing cell-associated Fas protein content in C13*, it failed to upregulate FasL and sFasL and induce apoptosis, irrespective of concentration and duration of CDDP treatment. In the resistant A2780-cp cells, neither Fas nor FasL upregulation and apoptosis were evident in the presence of CDDP. Addition of concentrated spent media from OV2008 after CDDP on C13* cells demonstrates a low level of apoptotic activity which is partially blocked by a Fas antagonistic Ab. Activation of the Fas signaling pathway, by addition to the cultures an agonistic Fas mAb, was effective in inducing apoptosis in both OV2008 and C13*. A significant interaction between CDDP and Fas agonist mAb was observed in the apoptotic response in OV2008 and C13* when cultured in the presence of both agents. Caspase-3 and -8 were cleaved into their fragments in a concentration- and time-dependent fashion after CDDP treatment in OV2008, but not C13* cells since apoptosis was not evident in these cells. Blocking the Fas receptor using a Fas antagonistic Ab only partially suppressed CDDP-induced apoptosis. Immunohistochemistry of human ovarian epithelial carcinomas reveals the

presence of Fas in low abundance in proliferatively active cells but in high levels in quiescent ones. Taken together, these data suggest that the dysregulation of the Fas/FasL would not significantly contribute to CDDP-resistance in ovarian epithelial cancer cells. However, our results are supportive of the notion that combined immuno- and chemo-therapy (i.e. agonistic Fas mAb plus CDDP) may provide added benefits in the treatment of both chemo-sensitive and -resistant ovarian tumors.

3.0 TABLE OF CONTENTS

1.0 ACKNOWLEDGEMENTS 2
2.0 ABSTRACT 3
3.0 TABLE OF CONTENTS 5
4.0 LIST OF ABBREVIATIONS 9
5.0 LIST OF FIGURES 11
6.0 LIST OF TABLES 13
7.0 INTRODUCTION 14
8.0 LITERATURE REVIEW 17
8.1 The Anatomy of the Mammalian Ovary 17
8.2 Changes in ovarian structure and function during development and ovulation 19
8.2.1 Ovarian Follicles 19
8.2.2 Ovarian Surface Epithelium 22
8.3 Human Ovarian Cancer 23
8.3.1 Ovarian Epithelial Cancer 24
8.3.2 Histological Types 25
8.3.3 Oncogenes and Regulatory factors in hOSE cancer 27
8.3.3.1 Endocrine, Paracrine and Autocrine Factors 28
8.3.3.2 Protein Kinases 28
8.3.3.3 GTP-Binding and Nuclear Proteins 30
8.3.3.4 Tumor-Suppressor Genes in hOSE Cancer Cells 31
8.4 Treatment of Ovarian Cancer 32
8.4.1 Surgery 32
8.4.2 Radiation Therapy 33

8.4.3 Chemotherapy 33

8.4.3.1 Cisplatin 35

8.5 Chemotherapy Resistance 37

8.5.1 Altered Drug Target 37

8.5.2 Drug Inactivation 38

8.5.3 Increased DNA Repair Activity 39

8.5.4 Increased DNA Damage Tolerance 40

8.6 CDDP Resistance 41

8.6.1 CDDP Accumulation and Inactivation 41

8.6.2 CDDP Resistance and DNA Repair 42

8.7 Apoptosis 43

8.7.1 Physiological Significance of Apoptosis 43

8.7.2 Morphological Aspects of Apoptosis 43

8.7.3 Biochemical Aspects of Apoptosis 44

8.7.4 Molecular Aspects of Apoptosis 46

8.7.5 Apoptosis in Cancer 52

8.7.6 Apoptosis and CDDP Resistance 52

8.8 The Fas and Fas Ligand System 54

8.8.1 Fas 54

8.8.2 Fas Ligand (Fas L) 56

8.8.3 The Fas and FasL system and cancer 59

9.0 HYPOTHESIS 61

10.0 OBJECTIVES 62

11.0 SPECIFIC AIMS 63

12.0 MATERIALS 64

13.0 METHODS 66
13.1 Ovarian Cancer Cell Culture 66
13.2 Biochemical Assessment of Apoptosis (DNA Ladders) 66
13.2.1 DNA Extraction and Quantification 66
13.2.2 DNA 3' -end labelling 67
13.2.3 Agarose Gel Electrophoresis and Autoradiography 67
13.2.4 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) 68
13.3 Morphological Assessment of Apoptosis 68
13.3.1 Phase Contrast Microscopy 68
13.3.2 Hoechst Staining of Cultured Cells 69
13.4 Protein Analysis 69
13.4.1 Protein Extraction and Quantification 69
13.4.2 Western Blot Analysis 70
13.5 Soluble FasL Activity 70
13.6 Preparation of human ovarian epithelial carcinoma 71
13.7 Immunohistochemistry 71
13.8 Statistical Analysis 72
14.0 RESULTS 73
14.1 Serum Deprivation 73
14.2 Morphologically Healthy vs Apoptotic hOSE cancer cells 73
14.3 CDDP Increased Fas and FasL Protein Contents 80
14.4 CDDP increased sFasL levels in OV2008 but not C13* hOSE cancer cell cultures 80
14.5 The effects of CDDP on Fas, FasL, sFas in A2780-s and A2780-cp hOSE cancer cells 87

14.6 CDDP and agonistic Fas Ab exhibit a synergistic effect on apopotosis _______90

14.7 CDDP-induced apoptosis is accompanied by caspase-8 cleavage ___________90

14.8 CDDP-induced apoptosis is accompanied by caspase-3 cleavage in OV2008 hOSE cancer cells. ______92

14.9 Antagonistic Fas Ab partially blocked apoptosis induced by CDDP. _________99

14.10 The presence of Fas and FasL protein in Ovarian Carcinoma Tissue ______102

15.0 DISCUSSION ______106

15.1 Experimental Model ______106

15.2 The influence of CDDP on Fas and FasL expression and apoptosis on Ovarian cancer cells. ______108

15.3 Possible Role of the Fas/FasL system in CDDP-induced apoptosis in hOSE cancer cells. ______110

15.4 A Possible Role of the Fas/FasL System in Chemoresistance. ___________113

15.5 Conclusions ______117

16.0 REFERENCES ______121

17.0 BIBLIOGRAPHY ______144

4.0 LIST OF ABBREVIATIONS

$\alpha[^{32}P]$-dCTP	deoxy cytosine tri-phosphate
Ab	antibody
AGE	agarose gel electrophoresis
Apaf-1	apoptosis protease activating factor-1
bp	base pair
CAD	caspase-activated DNase
CDDP	*cis*-Diammiinedichloroplatinum (II)
Ci	curie
CSF-1	colony-stimulating factor-1
Cyt c	cytochrome c
dCTP	deoxycytidine 5' -triphosphate
DD	death domain
DED	death effector domain
DFF	DNA fragmentation factor
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DTT	dithiotherietol
DXR	doxorubicin
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	fetal bovine serum
FLICE	FADD-interleukin-1β-converting enzyme (caspase-8)
GLB	Gel loading buffer
GSH	glutathione
HMG	high mobility group
hOSE	human ovarian surface epithelium
HRP	Horseradish peroxidase
ICE	interleukin-1 β- converting enzyme
IL-1 /IL-6	interleukin-1 and -6
IGF-1	Insulin-like Growth Factor-1
IgG	immunoglobulin G
IgM	immunoglobulin M
INF-α	interferon alpha
INF-β	interferon beta
IU	international unit
kb	kilo base
kDa	kilodalton
LMW	low molecular weight
MDR	multi-drug resistance

MMR	mismatch repair
NER	nucleotide excision repair
OSE	ovarian surface epithelium
PARP	poly (ADP)-ribose polymerase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
SEM	standard error of mean
SDS	sodium doecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween 20
p53	Tumor Suppressor p53
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGFα	Transforming Growth Factor alpha
TNFα	Tumor Necrosis Factor alpha
vs	versus

5.0 LIST OF FIGURES

Figure 1. Anatomy of the ovary ______ 18

Figure 2. The morphological features of apoptosis ______ 47

Figure 3. Fas and Fas ligand signaling system. ______ 58

Figure 4. Serum deprivation does not cause hOSE cancer cells to undergo apoptosis. _74

Figure 5. Phase contrast photomicrograph and assessment of nuclear morphology in OV2008 and C13* hOSE cancer cells. ______ 75

Figure 6. Concentration- and time-dependent apoptosis after CDDP treatment. ______ 77

Figure 7. Effect of CDDP and agonistic Fas Ab on cell number in OV2008 hOSE cancer cells. ______ 78

Figure 8. Basal Levels of Fas and FasL in OV2008 (sensitive) and C13*(resistant) hOSE Cancer Cells. ______ 79

Figure 9. Concentration dependent effect of CDDP on Fas and FasL protein contents._81

Figure 10. Time-dependent effect of CDDP on Fas protein levels in OV2008 and C13* hOSE cancer cells. ______ 82

Figure 11. Time-dependent effect of CDDP on FasL protein levels in OV2008 and C13* hOSE cancer cells ______ 83

Figure 12. Concentration-dependent effects of CDDP on soluble FasL and Fas protein levels in hOSE cancer cells cultures. ______ 85

Figure 13. Filtration vs Filtration and Ultracentrifugation on FasL in spent media OV2008 cultures ______ 86

Figure 14. Assessment of sFasL activity on C13* hOSE cancer cells ______ 88

Figure 15. Effects of CDDP on Fas, FasL and sFasL in A2780-s and A2780-cp hOSE cancer cells. ______ 89

Figure 16. Effects of CDDP and Agonistic Fas Ab on apoptosis in OV2008 and C13* hOSE cancer cells. ______ 91

Figure 17. Effect of different concentrations of CDDP on the cleavage of caspase-8 in OV2008 and C13* hOSE cancer cells. ______ 93

Figure 18. Time Course of cleavage of caspase-8 in OV2008 hOSE cancer cells induced by CDDP. ____ 94

Figure 19. Lack of effect of CDDP on caspase-8 cleavage in C13* hOSE cancer cells. _95

Figure 20. Concentration-dependent effects of CDDP on caspase-3 cleavage in OV2008 and C13* hOSE cancer cells. ____ 96

Figure 21. Temporal effects of CDDP on caspase-3 cleavage in OV2008 hOSE Cancer cells. ____ 97

Figure 22. Lack of effect of CDDP on caspase-3 cleavage in the CDDP-resistant cell line (C13*). ____ 98

Figure 23. Antagonistic Fas Ab effectively blocked apoptosis induced by agonistic Fas Ab in OV2008 and C13* hOSE cancer cells. ____ 100

Figure 24. Antagonistic Fas Ab effectively blocked caspase-3 cleavage induced by agonistic Fas Ab in OV2008 and C13* hOSE cancer cells. ____ 101

Figure 25. Blockade of the Fas Receptor partially reduced CDDP-induced apoptosis in OV2008 hOSE cancer cells. ____ 103

Figure 26. Detection of Fas/FasL, PCNA and cell death in human ovarian carcinomas. 104

Figure 27. Model ____ 119

6.0 LIST OF TABLES

Table 1 Comparisons of ovarian cancers 26

Table 2 Members of the caspase family and their known substrates 49

7.0 INTRODUCTION

Ovarian cancer is the most lethal gynecological cancer in the Western world and ranks fifth among the most common female cancers. Epithelial ovarian tumors account for about 90% of all human ovarian malignancies and originate from the simple epithelium covering the surface of the ovary (Nicosia and Nicosia 1988). Recent studies have suggested that cancer is characterised by enhanced cell proliferation and reduced physiological cell death, often referred to as apoptosis. Irradiation and many chemotherapeutic agents can activate the apoptotic death program in susceptible target cells, including epithelial ovarian cancer cells (Ormerod et al 1996; Soldatenkov et al 1995; Havrilesky et al 1995; Anthoney et al 1996).

CDDP is a widely used anti-cancer agent with a broad range of antitumour activity. The activity of CDDP is thought to be due to its ability to form inter- and intra-strand DNA crosslinks (Sherman and Lippard 1987) and its cytotoxic effect appears to result from inhibition of replication by CDDP-DNA adducts and G2 arrest, with subsequent induction of apoptosis (Sorenson et al 1990). Although cisplatin (*cis*-diamminedichloroplatinum II; CDDP) derivatives (e.g. carboplatin) and paclitaxel (taxol) are first line chemotherapeutic agents for the treatment of ovarian epithelial cancer, chemoresistance is a major therapeutic problem and the molecular mechanisms involved are poorly understood. The development of resistance may be cell-type specific and related to the dosing schedule (Andrews and Howell 1990). The mechanisms of chemoresistance appear to be multifactorial and are generally thought of in terms of altered pharmacodynamics and gene expression (e.g. MDR gene), modified drug target, increased rate of DNA repair and decreased rate of drug-induced DNA or macromolecule damage (Andrews and Howell 1990; Stewart et al 1996; Reed et al 1996). While our

knowledge on the events leading to chemoresistance is incomplete, failure to activate apoptosis in these cancer cells may confer resistance to these agents (Perego et al 1996).

Caspases are proteases that exist in the cell as proenzymes and have an absolute requirement for cleavage after aspartic acid. In apoptosis, caspases function in both cell disassembly (effectors) and in initiating this disassembly (initiators) (Thornberry and Lazebnik, 1998). The main effector caspase seems to be caspase-3, although the cell does not rely solely on this caspase since caspase-3 deficient mice do not display defects in apoptosis in all cell types (Kuida et al 1996). This indicates that other caspases can be activated and that there is much redundancy in this system. Caspases –2,-8,-9 and -10 seem to be initiators and can activate effector caspases such as caspase –2,-6,-7 and -3. General caspase inhibitors have been found to delay cell death induced by a variety of chemotherapeutic agents (Gamen et al 1997; Tolomeo et al 1998). Caspases are involved in mediating apoptosis induced by these agents, however, through which pathways these proteases are activated in response to these drugs are not known. One possible pathway may involve the cell death receptor (Fas) and its ligand [Fas Ligand (FasL)].

Fas is a cysteine-rich transmembrane glycoprotein which belongs to the tumour necrosis factor (TNF)/nerve growth factor receptor superfamily (Itoh et al 1991). Upon ligand binding, Fas induces apoptosis via the action of caspases, including caspase-8 and -3. FasL has been cloned (Suda and Nagata 1994) and is a type II transmembrane protein belonging to the TNF family. Soluble forms for both Fas and FasL exist. Soluble Fas (sFas) is generated by alternative splicing (Cheng et al 1994) while sFasL is generted via metalloproteinase cleavage of membrane bound FasL (Tanaka et al 1996). While the Fas/FasL system is believed to play an important role in the regulation of ovarian follicular atresia (Kim et al 1998), the expression of Fas has recently been reported in ovarian epithelial cancer cell lines (Uslu et al 1996; Wakahara et al 1997) and shown to be upregulated by CDDP (Uslu et al 1996). In fact, upregulation of Fas by chemotherapeutic agents occurs in many cell types. The involvement of the Fas system

in CDDP-induced apoptosis has not been clearly demonstrated. However the Fas system seems to be involved in a variety of other chemotherapeutic agents although the importance of their involvement seems in some cases to be cell type specific. If the Fas system is important for CDDP-induced apoptosis, dysregulation of this system could potentially lead to CDDP resistance. Many reports have demonstrated the lack of FasL upregulation to doxorubicin in drug resistant cells (Friesen et al 1997). In addition, adriamycin and doxorubicin resistant cells lack cell-surface Fas expression (Cai et al 1996; Fulda et al 1998). However, the role and regulation of the Fas/ FasL system in CDDP-resistance in epithelial ovarian cancer remains unclear.

In the present studies, we have examined the regulation of Fas and FasL expression in CDDP-mediated apoptosis in human ovarian surface epithelial cancer cells (OV2008/C13*/A2780-s/A2780-cp) and their possible role in CDDP-resistance. These studies demonstrate that increases in membrane bound Fas, FasL, sFasL and cleavage of caspase-3 and -8 following CDDP challenge are characteristic of CDDP-sensitive cells. Both CDDP-resistant cells fail to upregulate FasL and sFasL, and the A2780-cp CDDP-resistant cell line also failed to upregulate Fas. However, blocking the Fas receptor with an antagonistic antibody only partially attenuates CDDP-induced apoptosis in ovarian cancer cells, suggesting that other cell death pathway(s) may also be involved.

8.0 LITERATURE REVIEW

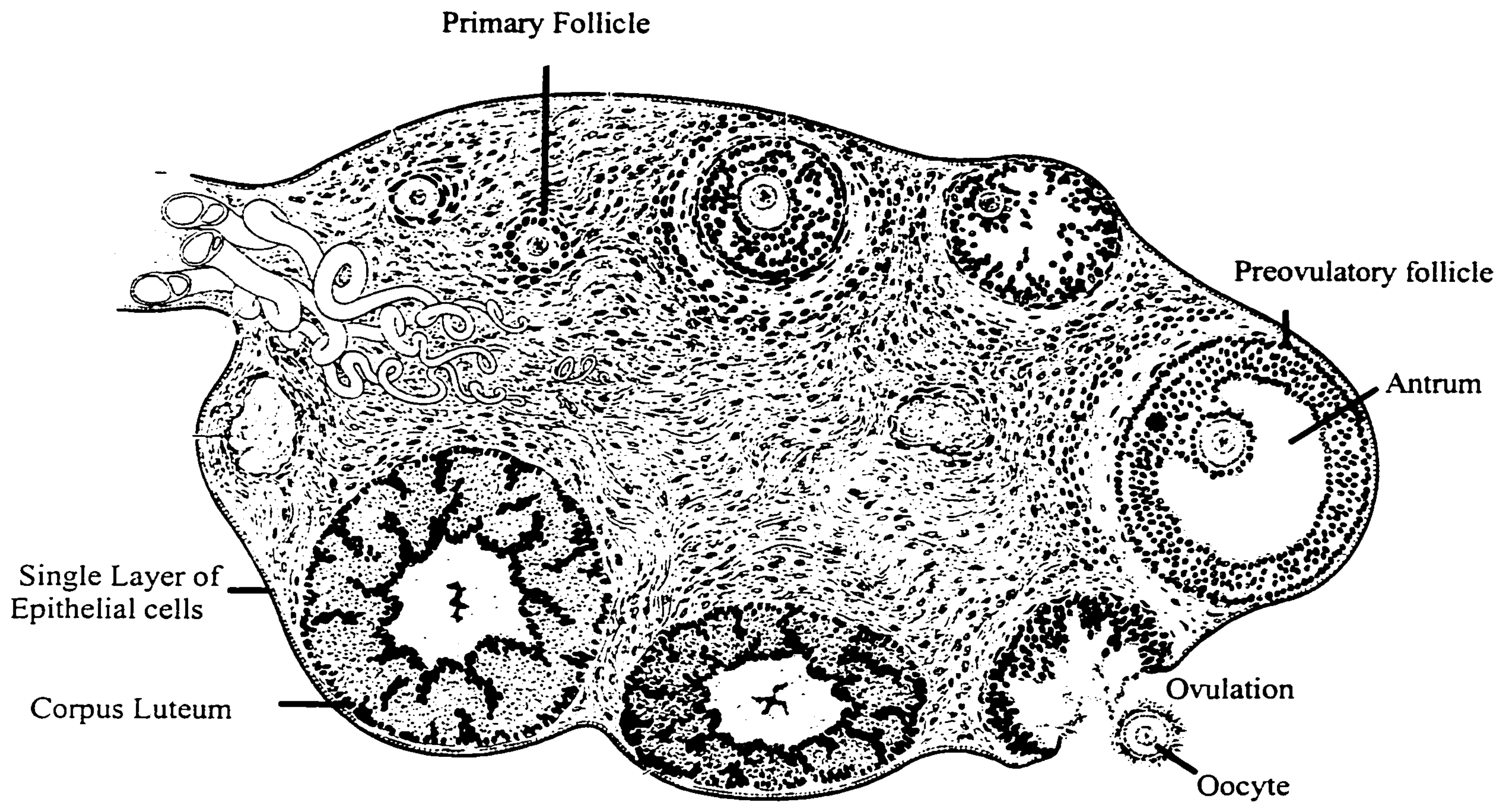
8.1 The Anatomy of the Mammalian Ovary

The ovary consists of three distinct, structurally heterogenous regions: an inner hilum at the point of attachment of the ovary to the mesovarium, a central medulla and an outer cortex. The hilum contains nerves, blood vessels and supporting connective tissue. The medulla consists of a heterogenous collection of cells that remain after the oocyte is ovulated. The cortex, which consists of immature germ cells (oocytes) enclosed in cellular complexes imbedded in the stroma (the follicles) is covered with coelomic epithelium, called the germinal epithelium (Figure 1).

The follicle is the basic functional unit of the ovary. The oocyte is situated in the centre of a primary follicle. As the follicle grows, it develops a fluid filled antrum and the oocyte projects into the antrum on a stalk of cumulus granulosa cells. The inner wall of the follicle is delineated by a basement membrane (lamina propria) which is covered by a stratified epithelium of membrana granulosa cells several layers thick. Theca cells form layers outside the basement membrane and are divided into the theca interna and externa. As the follicle grows, the basement membrane must be continuously remodeled and synthesized to accommodate the increasing size of the structure. The theca interna has a vascular supply that forms an anastomotic network terminating adjacent to the basement membrane so that the granulosa layer does not have a direct blood supply and instead depends on the passage of nutrients through the basement membrane. Theca cells are the major source of androgens in the follicle and therefore play an important role in the regulation of steroid production (Gore-Langton and Armstrong 1988). The outermost layers of the follicle are composed of fibroblast and smooth muscle fusiform cells making up the theca externa.

Figure 1. **Anatomy of the ovary**

The ovary is covered by a single layer of epithelial cells. Within the ovary, the follicles are present at different stages of development. The preovulatory follicle contains the oocyte which projects into the antrum on a stalk of granulosa cells. Granulosa cells line the antral cavity of the follicle and are seperated from the theca cell layer by the basement membrane.



modified from Ross and Schreiber 1986

The ovarian surface epithelium (OSE) is a simple squamous-to-cuboidal epithelium characterized by low-molecular weight keratins, simple desmosomes, apical microvilli, and a basal lamina. The OSE are supported by a basal lamina located along the border of the tunica albuginea. OSE cells have a mesodermal origin shared with the epithelia of the urogenital system and adrenal cortex. During early embryonic development, the mesoderm segregates into pluripotent mesenchyme and coelomic epithelium. OSE differentiates after invagination of the coelomic mesothelium over the developing gonadal ridges (Nicosia 1983). Thus, OSE cells are developmentally related to the underlying stromal fibroblasts. Coelomic epithelial cells penetrate into the fetal ovary and contribute to the development of granulosa cells. Furthermore, the gonadal ridge lies near the region where invagination of the coelomic epithelium gives rise to the Müllerian ducts. In the embryo, the coelomic epithelium in the gonadal region is competent to develop along many different pathways. Evidence of their developmental relationship to stromal fibroblasts are seen in vivo and in vitro: OSE contains vimentin filaments, which are typical for mesenchymal cells, and keratin (Auersperg et al 1994; Czernobilsky et al 1985). OSE produces not only epithelial (laminin and collagen IV) but also mesenchymal (collagen I and III) components of the extracellular matrix (Auersperg et al 1991) in response to a variety of environmental cues (Siemens and Auersperg 1988; Kruk et al 1994). During post-ovulatory repair, OSE cells reversibly modulate to a more fibroblast-like from. The simple phenotype of the adult OSE and its ability to modulate to a mesenhymal form indicate that these cells are relatively uncommitted and pleuripotent compared with other adult coelomic epithelial derivatives.

8.2 Changes in ovarian structure and function during development and ovulation

8.2.1 Ovarian Follicles

Formation of ovarian follicles begins during fetal development. Germ cells migrate to the genital ridge and colonize an area of mesoderm in the dorsal body wall where they interact with mesenchyme that will form the somatic components of the ovary (Hirshfield 1991). Germ cells stop dividing and enter meiosis, becoming arrested at the diplotene stage of the first meiotic division. These cells remain arrested at this stage until the follicle is recruited. Primordial follicle consists of an oocyte surrounded by a few flattened granulosa cells within a basement membrane.

Follicular development is commonly divided into distinct stages. Primordial follicles become primary follicles at the onset of growth, when the enlarged ooctye is surrounded by a single layer of cuboidal granulosa cells. A secondary follicle is characterized by multiple layers of granulosa cells. The appearance of an antrum indicates the conversion of the secondary follicle into a tertiary follicle. Near the end of this time, granulosa cells acquire receptors for LH, along with the theca cells, to respond to the ovulatory surge of LH. Following ovulation, the basement membrane breaks down completely, blood vessels invade the ruptured follicle and the granulosa and theca cells luteinize to form the corpus luteum (Lipner 1988). Luteinization involves extensive hypertrophy of the granulosa and theca cells, which develop increased cytoplasmic lipid droplets and produce large amounts of progesterone to prepare and sustain the uterine lining during pregnancy.

FSH is essential for the growth of the follicles to the preovulatory stage. Granulosa cells in preantral follicles express the receptor for FSH and, after expose to gonadotropin can produce estradiol, follicular fluid and LH receptor (Hirshfield 1991). FSH can then aid the follicle in the final stages of development, in preparation for ovulation and luteinization in response to the LH surge. Estrogen produced by the follicle exerts a negative feedback on FSH release from the pituitary. In addition, estrogen has local effects in the follicle to induce FSH receptors. The combination of

reduced circulating FSH and increased local FSH receptors results in the selection of those follicles that can produce sufficient estrogen (Hsueh et al 1984).

FSH receptors are only found on granulosa cells, while LH receptors are found on granulosa cells of preovulatory follicles and on theca cells of all follicles. These two gonadotropins are the major regulatory hormones for the synthesis of steroids on the ovary. Estrogen is derived from androgens by the aromatization of testosterone by aromatase. The major effect of FSH on granulosa cells early in follicular development is to induce aromatase activity, thus enabling the synthesis of estrogen. Upon stimulation by LH, the main steroidogenic function of theca cells is the synthesis of androgens, which are the rate-limiting substrates for granulosa cell estrogen production (Gore-Langton and Armstrong 1988). The combined action of FSH on granulosa cells and LH on theca cells is necessary for the production of estrogens. This interaction is known as the "two-cell, two-gonadotropin " theory for the control of estrogen production.

With the onset of menopause the ovary becomes the postmenopausal ovary; an atrophic, yellowish, lusterless structure with a wrinkled surface, despite high circulating levels of gonadotropins. Microscopically, the cortex is thin and usually devoid of follicles. Occasionally, the cortex shows evidence of stromal hyperplasia, which may result in the production of high levels of androgen (Ross and Schreiber, 1986). The ovary still produces androstenedione and testosterone by interstitial cells morphologically similar to testicular Leydig cells. The postmenopausal ovary does not secrete significant amounts of estrogens, and the major estrogen in the blood of postmenopausal women is estrone, thought to be derived from peripheral aromatization of adrenal androstenedione. The incidence of ovarian cancers is highest amoung postmenopausal women. In fact, development of ovarian cancers has been thought to be related to excessive gonadotropin production associated with the onset of menopause (Cramer and Welch, 1983).

8.2.2 Ovarian Surface Epithelium

Recent studies have shown that the OSE is far more complicated and physiologically versatile than would be predicted from its simple appearance. In tissue culture, the extracellular matrix (ECM) profoundly influences the phenotype of the cells and they, in turn, modulate ECM synthesis, lysis and physical restructuring. OSE cells can deposit epithelial as well as stromal ECM components, express integrins, collagens, fibronectin and vitronectin, and secrete chymotyrpsin-like and elastase-like peptidases, metalloproteinases and plasminogen activator inhibitor (Kruk et al 1992; Kruk et al 1994; Auersperg et al 1991). Thus OSE cells have the capacity to remodel the ovarian cortex through synthetic, physical, and proteolytic functions that may influence ovulation and the ovarian shrinkage and cyst formation that occur with age. The normal cells secrete and have receptors for agents with growth and differentiation capabilities. They produce TGF-β, which acts as a growth inhibitor, as well as the EGFR, which when stimulated by EGF is a potent mitogen (Berchuck et al 1992). TNFα induces both proliferation and inhibition and is expressed in OSE cells (Wu et al 1993). They also have receptors for ovarian steroids (Hamilton et al 1982) and gonadotropins (Zheng et al 1996) but their roles in normal OSE physiology is unknown. OSE cells secrete IL-1, IL-6, M-CSF, G-CSF and GM-CSF (Ziltener et al 1993). With the exception of G-CSF, all these factors are produced by ovarian cancer cells and stimulate their proliferation (Berchuck et al 1993). It is possible that secretion of cytokines is a normal function, but their recruitment into dysregulated autocrine loops may contribute to neoplastic progression.

Due to the preferential growth, the prospective preovulatory follicle protrudes onto the ovarian surface. Anatomical evidence suggests that OSE cells actively participate in the mechanics of gonadotropin-induced follicular rupture. Proteolytic enzymes released from the epithelial cells can degrade the basement membrane and underlying theca layer, thereby weakening the ovarian surface. The general consensus is

that degenerative epithelial cells are shed from the ovarian surface before ovulation (Murdoch et al 1995). Ovulation creates a wound at the ovarian surface. The defect is repaired by proliferation of epithelial cells from the perimeter of the ruptured follicle.

There are several reasons why the OSE may have an increased tendency for neoplasia. First, the surface epithelium is in close proximity to the paracrine influence of adjacent ovarian tissue and many of the receptors of the hormones and growth factors produced by the ovary are present on the OSE cells. As already mentioned, OSE cells are often found entrapped in the ovarian cortex and are potentially under increased mitotic pressure. Additionally, it may be significant that the surface epithelium shows delayed differentiation as manifested by its ability to differentiate along several pathways after transformation.

8.3 Human Ovarian Cancer

Ovarian cancer is the fifth most frequent cause of cancer in women. Relative prevalence increases with age and the majority of ovarian cancers are found in postmenopausal women. Incidence is highest in industrialized cultures with the exception of Japan. Circumstances that avert ovulation (oral contraceptive use, multiparty, lactation) protect against the development of ovarian cancer (Fathalla, 1971). Tumors of the ovary are either benign, borderline or malignant. Benign tumors require operative intervention but do not recur or metastasize, nor do they generally decrease survival. Borderline tumors, in contrast, are associated with a small risk of recurrence, may have invasive implants and thus may decrease survival. Malignant tumors of the ovary recur, metastasize, and decrease survival.

About 90% of all malignant tumors in the ovary are thought to arise from a single layer of epithelial cells that cover the ovarian surface, and approximately 10% of those are hereditary. Other histological types occur less frequently: 6% are sex-cord stromal

tumors, 3% germ cell tumors, and about 1% tumors of indeterminate histogenesis. The histologic type of tumor varies with age; most tumors in patients younger than 20 years are of germ cell origin, whereas most tumors in postmenopausal patients are of epithelial origin. Survival rates for most of these tumors are farily good (50-90 %; Williams et al 1997; Stenwig et al 1979). However, stage I ovarian epithelial carcinomas are asymptomatic and the cancer is usually advanced when diagnosis is made. As a result the survival rate is extremely low (30%).

8.3.1 Ovarian Epithelial Cancer

Cramer and Welch (1983) have proposed a model of tumorigenesis that attempts to reconcile the existing epidemiological and pathologic data regarding ovarian cancer. According to this model, the first step in tumorigenesis is the formation of epithelial inclusion cysts or invaginations. Fragments of OSE are displaced from the ovarian surface by adhesions or become trapped in the stroma as a result of ovulatory repair and changes in the ovarian contours with aging. Cyst formation would result in disruption of the connective tissue separating the OSE from the ovarian cortex. Thus the OSE cells would be in proximity to steroid producing cells, a microenvironment presumably favorable for proliferation and inhibition of growth arrest or apoptotic pathways. With neoplastic progression, tendency of OSE cells to express mesenchymal characteristics diminishes and the cells become increasingly committed to an epithelial phenotype.

OSE cells on the surface of the ovary express mesenchymal and epithelial characteristics, but epithelial differentiation predominates in cells within inclusion cysts (Radisavljevic 1976). The OSE cells modulate from an epithelial to a mesenchymal form *in vitro*, whereas cancer cells retain an epithelial morphology, as well as keratin, micorvilli, CA125 and E-cadherin (Auersperg et al 1994). In addition, an increased commitment of OSE cells to an epithelial phenotype has been reported in women with a

familial history of ovarian cancer (Auersperg et al 1997). Repetitive injury and repair of the OSE might provide additional proliferative stimuli or inhibition of cell cycle arrest signals and contribute to the formation of inclusion cysts. Growth factors (e.g EGF) in follicular fluid or released from platelets could contribute to this process. Inheritence of deleted or mutated genes is a possible basis for developing ovarian neoplasia due to ovulation. Induced genetic anomalies have been detected in rat OSE cells subjected to repetitive passages, mimicking the wound response and strengthening the proposal that continuous ovulations contributes to the pathogenesis of ovarian cancer (Godwin et al 1992). Genetically damaged cells that survive at the margins of ovulatory follicles may give rise to a malignant phenotype. The stimulus for malignant transformation however, remains unclear.

Hereditary ovarian cancers account for 5-10% of all cases. Three hereditary ovarian cancer syndromes with autosomal dominance have been described: (1) site-specific ovarian cancer, (2) nonpolyposis colon and ovarian cancer and (3) breast and ovarian cancer (Auersperg et al 1997). Mutations in a gene involved in the breast and ovarian cancer syndrome (BRCA1) appear to be responsible for a high production of cancers in women (Xu and Solomon, 1996). At the cellular level, these include apical microvilli and cilia, junctional complexes, epithelial membrane antigens and secretory products including mucins and CA125. At the multicellular level, the tumors form polarized epithelia, papillae, cysts and glandular structures.

8.3.2 Histological Types

Table 1 shows the current classification system for epithelial ovarian tumors. These tumors are not uncommonly mixed, with two or more cell types coexisting in the same neoplasm. The epithelium, in becoming malignant, exhibits a variety of Müllerian-type differentiations. For example, the epithelium that characterizes serous tumors

Table 1 Comparisons of ovarian cancers

Type	Frequency of all tumours*	
Common epithelial	90%	
Serous		46%
Mucinous		36%
Endometrioid		8%
Clear cell		3%
Brenner		2%
Mixed		3%
Undifferentiated		2%
Germ cell	3%	
Sex-cord stromal	6%	

(Robboy et al 1988)

* Indented figures add to 100% and are subsets of common epithelial ovarian cancer

resembles the normal lining of the fallopian tube. Mucinous tumors usually resemble the typical mucinous cells lining the normal endocervix. Endometrioid tumor cells are mostly malignant, and usually resemble cells of the endometrium. Clear cell carcinomas, resembling endometrioid tumors and often difficult to be distinguished from serous tumors, are often partially cystic and contain tubules, glands, papillae and cysts lined by clear cells Brenner tumors are mostly large and cystic and closely resemble urothelial differentiation. Undifferentiated carcinomas are defined as epithelial tumors that are so poorly differentiated that they cannot be clearly classified (Robboy et al 1988).

8.3.3 Oncogenes and Regulatory factors in hOSE cancer

Cancer may be thought of in broad terms as a disease of genes, arising from a variety of genetic alterations. These include: recessive and dominant mutations produced through large rearrangements, loss of DNA, or single-point mutations, all of which lead to distortions of either the expression or biochemical functions of the proteins for which these genes encode. Vague symptoms that result in the late diagnosis of ovarian cancer severly hamper the ability to gain insights into the genes which cause this disease. The majority of ovarian cancer specimens are from late stage and possess a complexity of genetic changes (Godwin et al 1994), making it difficult to establish the initiating mutation, and order of the mutations during the course of the cancer. Cytogenetic mapping of somatic ovarian cancer cells have revealed diverse heterozygotic allelic losses and rearragements. Structural alterations involving human chromosomes 1, 3, 6, 7, 9, 11 and 17 are common (Auersperg et al 1998).

8.3.3.1 Endocrine, Paracrine and Autocrine Factors

Development of ovarian cancers has been related to excessive gonadotropin production associated with the onset of menopause (Cramer and Welch, 1983). Receptors for gonadotropic hormones have been localized to some cancer cell lines (Emons et al 1992; Isola et al 1994). Ovarian cysts and tumors developed in transgenic mice that overexpressed luteinizing hormone (Risma et al 1997). More than half of malignant tumors express receptors for progesterone, androgens, or estrogen, and some ovarian tumors can synthesize and metabolize sex steroid hormones.

Based primarily on *in vitro* studies with established cell lines, growth factors and cytokines that are present within the ovary and regulate ovarian function have been implicated in the advancement of epithelial ovarian carcinomas (Berchuck et al 1993; Ziltener et al 1993). PDGF, IGF-I, IL-1 and -6, TGF-α and TNF-α augmented ovarian cancer cell growth, whereas TGF-β and INF-β and -α inhibited cell proliferation (Berchuck et al 1993; Wu et al 1992) and downregulation of TGF-β, a strong inhibitor of epithelial cell growth, may contibute to the process of tumorigensis. While TGF-β inhibits proliferation of normal hOSE cells, it can induce apoptosis in addition to growth inhibition in ovarian cancers (Havrilesky et al 1995).

8.3.3.2 Protein Kinases

The protein kinases constitute the largest functional group of oncogenes. They can be grouped into two classes: serine/threonine protein kinases and tyrosine kinases. Deregulated protein kinase activity results in aberrant phosphorylation, often culminating in unregulated mitogenic response.

The c-*ERB*B1/EGFR gene encodes for a growth factor receptor with tyrosine kinase activity. This receptor is expressed in 54% of ovarian carcinomas although

overexpression of this gene is seen only in 9-17% of ovarian cancers (van Dam et al 1991). A mutated form of the EGFR (Type III receptor variant, EG-FRvIII) was found to be expressed in 73% of ovarian carcinomas, in 5 of 8 hOSE cancer cell lines but not in the normal ovary or primary cultures of hOSE cells (Moscatello et al 1995). The role of the EGFR and of this variant in oncogenesis is unclear. The *ERB*B2 (*HER-2/neu*) proto-oncogene is a cell surface receptor similar in structure to EGFR. Overexpression or amplification of this receptor have been found in 5-32% of ovarian cancers (Bast et al 1993). Amplification was more frequent in invasive rather than borderline tumours suggesting a limited role in early neoplastic transformation.

The c-MET proto-oncogene encodes a receptor tyrosine kinase for the hepatocyte growth factor (HGF). HGF is a potent mitogen for epithelial cells and promotes cell motility and invasion. It is expressed in normal OSE cells and benign tumors and is overexpressed in a subset of epithelial carcinomas (DiRenzo et al 1994). The carcinomas were generally of lower histologic grade and associated with stage I disease, suggesting that c-*MET* may act early on in progression of OSE cancers and may confer additional selective advantage to transformed cells.

The receptor for CSF-1 is a transmembrane protein tyrosine kinase encoded by the proto-oncogene *FMS*. CSF-1 is a mitogen and activator of circulating monocytes and tissue macrophages. Expression of CSF-1 is frequent in ovarian cancers and corrleates with disease status (Kacinski et al 1990; Baiocchi et al 1991) The presence of *FMS* and CSF-1 in the neoplastic epithelial cells suggest the existence of a potential autocrine loop. Plasma levels of CSF-1 have corrleated with active or recurrent disease (Kacinski et al 1989).

A number of protein serine/threonine kinases have been found altered in human malignancies. The expression of the cellular proto-oncogene *AKT2*, a serine-threonine protein kinase related to protein kinase C, has been found to be altered in ovarian neoplasms. AKT2 is amplified or overexpressed in 12-25% of aggressive malignant

ovarian tumors. These particular tumours tend to have a poorer prognosis (Cheng et al 1992). Phosphatidylinositol 3-kinase (PI3 Kinase) is upstream of the AKT2 signaling pathway. The copy number of its catalytic subunit is frequently increased in ovarian cancers and is associated with increased activity leading to proliferation (Shayesteh et al 1999).

8.3.3.3 GTP-Binding and Nuclear Proteins

The GTP-binding proteins represent a class of proto-oncogenes that function to couple messages from receptor kinases to intracellular secondary messengers. Cell signaling by growth factor receptors is mediated through an elaborate cascade of molecules that must ultimately reach their nucleus in order to activate and regulate gene transcription. Several proto-oncogenes encode proteins that are localized in the cell nucleus and are believed to act as transcription factors.

The *RAS* gene family represents one of the most frequently mutated oncogenes in human malignancy. In ovarian cancer, alterations of the Ki-*RAS* gene are the most prevalent. The highest percentage of alterations in Ki-*RAS* appear in 26-75% of mucinous tumours. In contrast, expression of *RAS* was not observed in normal ovarian control cases (Yaginuma et al 1992). It has been reported that the *MYC* gene, which encodes a nuclear transcription factor, is amplified in 25% of malignant ovarian tumours (Baker et al 1990). Amplification and/or overexpression of this gene has predominantly been associated with more aggressive late stage or high grade tumors. This association brings into question whether *MYC* alterations are a contributing factor to disease or just a random event that occurs in advanced stage tumours due to aneuploidy in the tumor.

8.3.3.4 Tumor-Suppressor Genes in hOSE Cancer Cells

Of all the dominantly acting oncogenes and recessive tumor-suppressor genes, p53 gene is most frequently mutated in human cancers. The p53 gene product was originally detected by virtue of its ability to form a stable complex with the SV40 large T antigen (Soussi et al 1990). The gene encodes a nuclear phosphoprotein which exists at low levels in virtually all normal cells. Wild-type p53 acts as a negative regulator of cell growth and is induced following DNA damage and mediates cell-cycle arrest in late G1 and to a lesser extent in G2. In some contexts, wild-type p53 can induce apoptosis and the absence of the wild-type protein leads to resistance to ionizing radiation and chemotherapeutic agents (Perego et al 1996). Wild-type p53 appears to mediate growth supression in part through its specific DNA-binding and transcriptional regulatory abilities. In particular, wild-type p53 can enhance the expression of p21/WAF-11/CIP1, a protein capable of inhibiting cyclin-dependent kinases and arresting cell division (El-Deiry et al 1993). Mutant forms of p53 no longer possess the ability to arrest cell growth and to induce apoptosis. To date, more than 135 ovarian cancers have been found to carry mutations in the p53 gene, although the majority of these tumors are late-stage, high-grade epithelial tumors of the ovary. p53 overexpression was found to be associated with an aneuploid DNA content but did not correlate with age, grade or median survival (Marks et al 1991). p53 mutations have been observed in 37-80% of the advanced epithelial ovarian cancers studied and between 16-40% of early ovarian cancers (Marks et al 1991; Kohler et al 1993). The lower frequency of p53 mutations in cancers confined to the ovary and the near absence of mutations in benign and borderline ovarian neoplasms suggest that p53 alterations may be a relatively late event in the development of the malignant phenotype.

Mutations in the human breast and ovarian tumor suppressor gene BRCA1 are found in 50-80% of familial cancers but in only a small percentage of sporadic tumors

(Takahashi et al 1995). Reports on its function have been contradictory, but the protein structure suggests that it is a nuclear transcription factor. It is found in rapidly proliferating cells undergoing differentiation and expression occurs mainly in the testis, thymus, ovary and breast. (Marquis et al 1995). Recently, it was found that BRCA1 and human Rad51 (key component in double-stranded DNA repair pathway) colocalize and interact physically in S-phase cells, thus supporting a role for BRCA1 in DNA damage repair (Scully et al 1997). In addition, in patients with a familial ovarian cancer there may be an altered or defective capacity of OSE cells to take part in repair process as demonstrated by the inability of these cells to produce extracellular matrix (Auersperg et al 1997).

8.4 Treatment of Ovarian Cancer

The selection of therapy for patients with epithelial ovarian tumours is based upon anatomic stage and the previously described clinico-pathologic features. Therapeutic options may include cytoreductive surgery, chemotherapy, radiotherapy, or a combination of these modalities. Only 10-15% of all patients with epithelial ovarian cancer are diagnosed with early-stage disease and approximately one-third of all cured patients are derived from Stages I and II (Ozols et al 1997). Despite decades of effort aimed at improving methods of early detection and diagnosis, the majority of cases of cancer of the ovary are not diagnosed until the disease is advanced and has spread beyond the ovary.

8.4.1 Surgery

Cytoreductive surgery involves removal of bulky tumor masses. Such effects are likely to increase the ability to tolerate the intensive chemotherapy that is required. More

importantly, removal of large tumor masses may enhance the response of the remaining tumor to chemotherapy. In fact, several studies have shown that there is an increase in survival rate in those patients under going chemotherapy or radiotherapy after their tumor masses were reduced to nonpalpable levels compared to those that remained palpable (Delclos and Smith, 1975).

8.4.2 Radiation Therapy

The aim of radiation therapy is to deliver a precisely measured dose of irradiation to a defined tumor volume with as minimal damage as possible to surrounding tissues. Sensitivity to radiation depends on the phase of the cell cycle and the duration of exposure of the tumour to the source of irradiation (Terasima and Tolmach 1963). Irradiation effects are apparent only when the cells are in a proliferative cycle and going through one or more mitoses. Since most ovarian cancers spread throughout the peritoneal cavity, radiation therapy for ovarian cancer should employ techniques that encompass the whole peritoneal cavity so as to attain maximal benefits. However, the dose of radiation that can be delivered safely to the abdomen is considered lower than that considered optimal for successful treatment of solid tumors. Radiation provides modest benefits to patients with a small number of residual clonogenic cells and there is little or no curative potential for pateints with bulky diseases (Perez et al 1997)

8.4.3 Chemotherapy

Cell cycle events have important implications for cancer chemotherapy. Most chemotherapeutic agents act primarily by disrupting some critical aspects of DNA, RNA or protein synthesis, which is most likely to be destructive to rapidly proliferating cells. The rate of tumour growth is dependent on the growth fraction and cell death. Growth

fraction is the fraction of cells in the tumor mass that are actively proliferating. Only a fraction of these cells in a typical tumor mass are rapidly proliferating; the rest are quiescent. In animal tumor models, chemotherapeutic agents act by killing a constant fraction rather than a constant number of the cells exposed to the drug. Only when the log kill is very large (>99%) and the therapies are repetitive can single agent chemotherapy be curative. Prolonged survival can only be achieved when the cell population is reduced to between 10^1 and 10^4 cells. Cell burdens of this size are not clinically detectable, emphasizing the importance of continuation of chemotherapy even if residual disease is not detectable (Young 1997).

Chemotherapy agents have a complex mechanism of action and generally affect tumor cells through several pathways. Certain anti-cancer drugs are cell-cycle specific and proliferation independent whereas others are not dependent on cell-cycle stage. Cell cycle specific agents depend on the proliferative fraction of the tumor. Hydroxy urea which inhibits ribonucleotide reductase is a typical example. In order to optimize the effectiveness of the drug, it is important to achieve a drug concentration and treatment duration effect at critical tumor sites. Drug binding, lipid solubility, blood supply to tumor and active membrane transport factors are important considerations for achieving adequate concentrations of drug. Some drugs (e.g cyclophosphamide) are administered as inactive compounds and are needed to be metabolized to an active form via the liver. Drug excretion is primarily achieved by the liver and kidneys. Although many effective anticancer agents exist, their curative potential depends on kinetics, drug access, tumor sensitivity and resistance.

Cancer chemotherapy is always a balance between the benefits derived from tumor destruction and the injury produced to the normal tissues. Bone marrow toxicity is the most common side effect of cytotoxic drugs. Gastrointestinal, alopecia, skin, neural and genitourinary toxicity are common side effects of chemotherapy, with prominence depending on drug action and mode of elimination (Young 1997).

8.4.3.1 Cisplatin

The biological activity of *cis*-diamminedichloroplatinum (II) (CDDP or cisplatin) was discovered accidentally during studies of growth inhibtion of *Escherichia coli* (Rosenberg et al 1967). Since this finding, CDDP has evolved into the mainstay of chemotherapy against ovarian cancer (Ozols et al 1997).

CDDP influx into the cell is believed to be by both active and passive mechansims (Ma et al 1998). Evidence for CDDP uptake by passive diffusion indicates that CDDP uptake is nonsaturable, even up to its solubility limit, and not inhibited by structural analogs (Gately et al 1993). CDDP accumulation into many cell types is partially energy dependent, oubain inhibitable, sodium dependent, and sensitive to changes in membrane potential and cAMP levels, indicating that a carrier-mediated transportor protein may be involved.

Activated CDDP is a potent electrophile that will react with any nucleophile, including the sulfhydryl groups on proteins and nucleophilic groups on nucleic acids. Several CDDP-DNA adducts have been identified, all occuring in the major groove of the DNA. The two major products are the intra-strand crosslinks cisPt$(NH_3)_2$d(pGpG) (1,2-d(GpG) and cisPt$(NH_3)_2$d(pApG) (1,2-d(ApG). These adducts represent 65% and 25%, of the total amount of adducts formed, respectively (Eastman, 1987). Minor adducts are monofunctionally CDDP bound to guanine, inter-strand crosslinks between two guanine nucleotides and intra-strand crosslinks between two guanines separated by one or more bases. Cytotoxicity has been believed to result from inhibition of DNA synthesis (Harder and Rosenberg 1970) and recent evidence suggests that the CDDP-induced DNA damage can lead to G_2 phase arrest and subsequently apoptosis (Sorenson et al 1990). The signal transduction mechansim that links DNA damage to the cell death pathway

remains unknown. There are proteins that recognize the conformational changes in damaged DNA (Bruhn et al 1992). The NER pathway can remove CDDP-adducts (1,2 and 1,3 intrastrand crosslinks) and one of the componenets of the gene product of XPA, is the damage recognition protein (Huang et al 1994; Asahina et al 1994). Once damaged DNA is recognized, it is unwound by the transcription factor TFIIH. Studies with cell extracts have demonstrated the existence of other proteins that bind to CDDP-damaged DNA. One such protein, termed the structure-specific recognition protein (SSRP), has the high mobility group (HMG) domain. These classes of proteins recognize DNA structural elements such as bends or CDDP-modified DNA (Chow et al 1994). However, it is believed that these HMG proteins may actually block the lesions from NER repair.

Following CDDP-induced DNA damage, the cell undergoes cycle arrest to allow repair of the damaged DNA. If it is irrepairable, the cell undergoes apoptosis, a process involving p53. In fact, DNA damage induced by CDDP can induce p53 (Zhan et al 1993). After DNA damage, the p53 protein is thought to bind to DNA and to activate multiple genes involved in apoptosis (e.g. bax, Fas, IGF-BP3). The upstream events leading to p53 induction are unknown but one may speculate that damage recognition proteins (DRPs) may provide the signal. Since p53 itself can bind to DNA ends and excision repair damage sites or internal deletion loops (Lee et al 1995), it is possible that both p53 and a damage-detector protein are localized at the site of DNA damage and repair where phosphorylation or other activating signals can then be processed. p53 can also bind to RNA polymerase II basal transcription factor, TFIIH, which is involved in the NER pathway (Wang et al 1995) and thus may be able to detect DNA damage through direct interaction with NER proteins.

Two genes that are regulated by p53 could influence the decision to activate the apoptotic pathway: bax and IGF-BP3. It is clear that overexpression of bcl-2 can block p53 mediated apoptosis. Bax binds to bcl-2 and antagonizes its abiltiy to block apoptosis.

IGF-BP3 blocks the IGF mitotic signaling pathway by binding to IGF and preventing its interaction with its receptor. It has been reported that p53 can also regulate Fas expression (Owen-Schaub et al 1995), although a p53 responsive element in the Fas gene has not as yet been demonstrated. Recent studies show that p53 can regulate Fas in a non-transcription dependent fashion by regulating its trafficking through the Golgi, thus increasing the number of receptors on the cell surface (Bennett et al 1998). In addition, CDDP treatment can result in JNK activation and protein kinase Cε translocation to the nucleus (Ohmori and Arteaga 1998) and these factors may be involved in CDDP cytotoxicity. Despite the success of CDDP in treating ovarian cancers, intrinsic resistance or acquired resistance is still a major clinical problem.

8.5 Chemotherapy Resistance

Multidrug resistance (MDR) is a major clinical problem in ovarian cancer and understanding the molecular basis is important for the design of new treatment strategies. MDR is known to be multifactorial, consisting of mechanisms that prevent cytotoxic drugs from reaching their targets and mechanisms that control cellular responses downstream of the drug/target interaction. The first group of resistance mechansims includes altered drug transport, increased drug inactivation and mutation or altered expression of drug targets. The second group consists of altered DNA repair activity and defects in the response to DNA damage.

8.5.1 Altered Drug Target

The ability of cells to increase the efflux of chemotherapeutic drugs often leads to decreased intracellular accummulation and consequently increased chemoresistance. Increased expression of an ATP-dependent drug efflux pump [P-glycoprotein (MDR1)],

can confer resistance to a wide variety of natural agents, but not alkylating or platinum drugs (Goldstein 1996). The role of MDR1 in ovarian cancers is presently unclear (Bourhis et al 1989; Holzmayer et al 1992). Another ATP-dependent multidrug transport protein, MRP has been shown to confer resistance to a series of drugs including doxorubicin, etoposide and daunorubicin (Cole et al 1992). The frequency of MRP expression in untreated ovarian cancers appears higher than that of MDR1 (Kavallaris et al 1996).

Glutathione (GSH), a tripeptide thiol, is a potent nucleophile that can react with alkylating agents and CDDP. This reaction forms a GSH-platinum complex that is then eliminated from the cell by an ATP-dependent pump, GS-X (Ishikawa and Ali-Osman, 1993). It is believed that GSH protects cells by intercepting reactive platinum complexes which otherwise react with DNA. GSH may also protect cells by supporting DNA repair, possibly by stabilizing repair enzymes such as DNA polymerase α (Lai et al 1989). Efforts to sensitize the tumours to chemotherapeutic agents by modulating the function of these pumps are underway (Tsuruo et al 1981).

8.5.2 Drug Inactivation

A wide variety of detoxication pathways exist that enable a cell to inactivate cytotoxic drugs. The mechanisms that have received the most attention in mediating drug resistance in ovarian cancer include increased levels of GSH, GSH-S transferases and metallothioneins. Inhibitors of GSH synthesis have been used to clarify the role of GSH in drug inactivation. However, the effect of the inhibitors on chemo-sensitivity has been unpredictable with respect to the various cell lines and platinum drugs examined (Hamilton et al 1985; O'Dwyer et al 1992). The GSH-S transferases (GST's), a multigene family which catalyzes the conjugation of GSH with some chemotherapeutic agents, has been implicated in resistance to alkylating agents, anthracyclines, and platinum drugs.

Overexpression and antisense studies support a role forGSTs in resistance, although conflicting data exist regarding the relationship between drug resistance and increased levels of activity of GSTs in ovarian cancer cell lines (Lewis et al 1988; Saburi et al 1989). Studies indicate that neither tumor GST activity nor expression level is predictive of response to chemotherapy in ovarian cancer patients (Auersperg et al 1998). Metallothioneins (MT) have been implicated in drug resistance to a variety of agents including CDDP and melphalan due to their presumed role in the detoxification of heavy metal ions. However, the association between increased MT levels and anticancer drug resistance in vitro remains unclear (Kelley et al 1988; Schilder et al 1990).

8.5.3 Increased DNA Repair Activity

Failure to prevent DNA damage by mechanisms such as those described above leaves a cell with no option but to repair or tolerate DNA damage if it is to survive. To protect and maintain the integrity of DNA, mammalian cells have developed a variety of mechanisms for repairing various types of DNA damage. As mentioned, the removal of bulky DNA adducts formed by chemotherapeutic drugs occurs predominantly by the NER pathway. Cell lines exhibiting mutations in repair genes exhibit hypersensitivity to DNA damaging agents. With regards to ovarian cancer, the majority of the studies involving DNA repair have focused on platinum-DNA adducts. Increased repair of Pt-DNA adducts has been shown to be associated with CDDP resistance in human ovarian cancer cell lines (Masuda et al 1990; Lai et al 1988). Although the molecular basis for increased DNA repair activity in resistant cells has not been fully defined, increased expression of a few putative DNA repair genes has been suggested to contribute to the enhanced repair capacity observed in some CDDP-resistant cells. Increased XPA and excision repair enzyme (ERCC1) mRNA levels have been observed in ovarian cancer tissues obtained from patients refractory to platinum drugs (Dabholkar et al 1994).

Modulation of resistance resulting from enhanced DNA repair activity has been limited by the availability of effective repair inhibitors.

8.5.4 Increased DNA Damage Tolerance

A frequently observed but currently unexplained feature found in MDR cells is the capacity to survive despite high levels of drug-induced damage. One tolerance mechanism that has been described for DNA damaging drugs, such as alkylating agents or platinum drugs, is enhanced capacity for replicative bypass. Enhanced replicative bypass, a process that allows a cell to continue DNA synthesis past a lesion, can enable a cell to successfully continue DNA synthesis and arrest in the G_2 phase of the cell cycle, after which postreplication repair processes can correct the DNA damage before mitosis. It has been shown in vitro that DNA polymerase can efficiently bypass a single intrastrand crosslink (Hoffman et al 1995).

The cellular resistance mechanisms described thus far act to reduce the overall level of drug-induced damage. If this threshold is exceeded, however, an intracellular signaling pathway may be activated that results in apoptosis. Since an intricate sequence of events is required for cell death to occur, it is possible that a mutation in one of the participating proteins of the pathway may enable a cell to survive increased amounts of damage. Alternatively, overexpression of a negative regulator of cell death in a resistant cell could also suppress apoptosis. Mutations in the p53 gene and overexpression of the *Bcl-2* oncogene are examples of each of these scenarios, respectively. In the presence of DNA damage, the wild-type p53 protein activates genes that are capable of inhibiting cell growth and/or inducing apoptosis. Overexpression of a dominant negative p53 mutant protein has been shown to reduce drug sensitivity in some cells, but not others, suggesting that p53-mediated cell death depends on other cellular factors (Lowe et al 1993; Fan et al 1995; Vasey et al 1996). Mutations in p53 are common in late stage

ovarian cancer. One study found a significant correlation between p53 accumulation, p53 mutation, and response to CDDP therapy (Righetti et al 1996). Overexpression of the *Bcl-2* and *Bcl-x* genes has been shown to protect cell from apoptotic death after exposure to a variety of drugs (Minn et al 1995; Miyashita and Reed 1993). However, in a recent ovarian cancer study, *Bcl-2* expression was not found to be closely associated with the response to chemotherapy (Herod et al 1996).

In addition to the direct disruption of programmed cell death pathways, factors that modulate the activity of signal transduction pathways can also affect the overall drug sensitivity or resistance of cells. The expression of certain protooncogenes such as H-Ras, v-abl and Her2/neu have been shown in some cases to promote the survival of cells after exposure to cytotoxic drugs (Isonishi et al 1991; Chapman et al 1995). Conversely, several agents (e.g tamoxifen, EGF, IL-1 and TNF-α) that activate or inhibit signal transduction pathways have been shown to enhance drug cytotoxicity in various cell lines (Kroning et al 1995; Auersperg et al 1998).

8.6 CDDP Resistance

8.6.1 CDDP Accumulation and Inactivation

Although the mechanisms of acquired resistance has been extensively studied, relatively little is known about intrinsic CDDP resistance. As already mentioned, potential mechanisms responsible for acquired resistance can be classified into two categories: those that lead to a reduction in the formation of cytotoxic DNA lesions and those that minimize their impact. A reduction in CDDP cellular accumulation is one mechanim used to reduce the formation of DNA lesions. CDDP influx is believed to be due to both active and passive mechanims. The active mechansim had been lost in a CDDP-resistant ovarian cancer cell line (Ma et al 1998). In a panel of 12 cell lines derived from ovarian tumors of patients with or without platinum-based chemotherapy,

CDDP accumulation varied five-fold but was not associated with CDDP sensitivity (Johnson et al 1996). However, when decreases in influx occured they were modest, even when the level of resistance was high (Lai et al 1989; Andrews et al 1985; Andrews et al 1987). The MDR1 pump is not involved in CDDP resistance (Deuchars and Ling 1989). GSH may protect cells by intercepting reactive platinum complexes, thus preventing their reaction with DNA. GSH may also protect cells by supporting DNA repair possibly by stabilizing repair enzymes such as DNA polymerase α (Lai et al 1989). Increased levels of GSH have been observed in many CDDP-resistant ovarian epithelial cancer cells (Hamilton et al 1985; Godwin et al 1992), which may be due to c-jun mediated overexpression of the enzyme γ-glutamyl cysteine synthetase (Yao et al 1995). Buthionine sulfoximine (BSO) irreversibly inhibits this enzyme and reduces the levels of GSH. However, CDDP resistance is not completely reversed by BSO, suggesting that there are other cellular mechanisms involved (Andrews et al 1985).

8.6.2 CDDP Resistance and DNA Repair

As mentioned, CDDP-damaged DNA can be repaired by many mechansims, two of which are the NER and mismatch repair (MMR) pathway. Increased repair of CDDP-DNA adducts has been demonstrated in many CDDP-resistant human ovarian epithelial cancer cells (Johnson et al 1994; Masuda et al 1990; Parker et al 1991). Relative levels of the proteins involved in the NER pathway have been measured in CDDP sensitive and resistant tumors. The XPA, XPE [general damage recognition proteins (DRP) in NER] and ERCC1 (involved in 5' incision) proteins were found to be overexpressed in CDDP-resistant ovarian cancer cells as well as cells of other origins (Chu and Chang 1990; Dabholkar et al 1992; Dabholkar et al 1994). In addition, research into repair of CDDP-adducts has demonstrated a higher repair rate in actively transcribed genes than in silent or non-coding regions of DNA (Johnson et al 1994). The MMR system seems to be

involved in G2 arrest rather than G1 arrest. Hence, G2 arrest most commonly seen with CDDP may be related to the MMR system (Mello et al 1996; Sorenson et al 1990). A CDDP resistant MMR-deficient cell line showed a decrease in apoptosis (Aebi et al 1996). The DRP of the MMR system, hMutα, which recognizes platinum-DNA adducts and triggers apoptosis (Duckett et al 1996), is overexpressed in human ovarian cancer cells as well as other tumors. It has been hypothesized that the hMSH2 (another DRP of the MMR pathway) shields CDDP-adducts and blocks MMR, thus allowing adducts to persist. The loss of the MMR would lead to enhanced repair of CDDP adducts by the NER and increased resistance. JNK regulates DNA repair and inhibition of this pathway sensitizes tumor cells to CDDP, suggesting that the increase in JNK and c-jun in response to CDDP may enhance DNA repair.

8.7 Apoptosis

8.7.1 Physiological Significance of Apoptosis

Apoptosis (or programmed cell death) is a normal, physiological process of cell death (Wyllie et al 1980; Kerr et al 1972) It is a genetically controlled process by which unwanted cells are eliminated without eliciting an immunological response. It has been postulated that all differentiated cells in multicellular organisms are capable of undergoing apoptosis through a highly conserved suicidal program (Raff, 1992; Raff et al 1993; Thompson, 1995). The apoptotic death programme may be activated by deprivation of survival factors, binding an apoptosis-inducing ligand to its receptor or irreversible damage (e.g DNA damaging agents) to cellular macromolecules (Jacobson et al 1997; Hsueh et al 1994).

8.7.2 Morphological Aspects of Apoptosis

Early in the apoptotic process the cell loses its volume (cytoplasmic condensation) and pulls away from its neighbouring cells. Blebs are formed at the cell membrane while the chromatin in the nucleus condenses and migrates to the nuclear envelope. The other organelles (e.g. mitochondria, golgi, etc.) retain their structure. In the final stages of apoptosis, the nucleus disintegrates into spherical dense fragments and the cell begins to fragment as the membrane blebs lead to budding off of membrane-bound vesicles (apoptotic bodies; Kerr et al, 1972; Kerr et al, 1994). Apoptotic bodies containing cytoplasmic and nuclear material of the dying cell, are quickly removed by neighbouring cells via phagocytosis (Savill, 1995). Thus, no cellular contents spill into the extracellular space and, as a consequence, no immunological response is elicited (Figure 2).

In contrast, necrotic cell death affects a continuous tract of cells in a tissue. Necrosis is a consequence of physical injury or traumatisation, such as an ischemic insult. This leads to loss of cell structure and swelling, as the cell loses its ability to maintain the ionic potential across its cell membrane, and ultimately cell rupture. The cytoplasmic contents are released into the extracellular space resulting in an immune response and inflammation.

8.7.3 Biochemical Aspects of Apoptosis

Apoptosis is an active, energy-utilising, gene directed process. Synthesis of new mRNA and protein appears to be required for apoptotic cell death in some but not all cells (Martin et al 1988; Schwartz and Osborne 1993). A biochemical hallmark of apoptosis is the cleavage of chromosomal DNA into nucleosomal units (180 bp multiples), which appears to be the final event in the cell death process (Wyllie et al 1984). The Ca2+ /Mg2+-dependent endonuclease DNaseI may be one of the enzymes responsible for DNA degradation (Boone and Tsang, 1997). Another endonuclease responsible for this

genomic degradation has been cloned in the mouse and is a caspase-activated DNase (CAD) which is complexed to its inhibitor ICAD. The human homologue of ICAD is DNA fragmentation factor (DFF). Both ICAD and DFF can be cleaved by caspase-3. Cleavage of ICAD releases CAD, which translocates into the nucleus and cleaves the DNA, whereas DFF enters the nucleus after cleavage and presumably activates a nuclear DNase similar to CAD (Enari et al 1998; Liu et al 1997). Also, DNA fragmentation does not always produce the 180 bp multiples but much larger 300 kb multiples, a size associated with chromatin loops (Ormerod et al 1994). In contrast, necrotic cell death is passive, does not use ATP nor involve new protein or RNA synthesis. The breakdown of DNA in necrotic cells is random and generalized, resulting in a 'smear' when the DNA is resolved on agarose gels (Afans'ev et al 1986).

Type II transglutaminase is a Ca2+-dependent cytosolic protein which is selectively upregulated in cells undergoing apoptosis (Piacentini et al 1994). During apoptosis, the large elevation of cytoplasmic calcium is sufficient to activate the transglutaminase which catalyses irreversible cross links between the glutamine residue of one protein and the lysine residue of another (Piacentini et al 1994). Cross linking of intracellular proteins stabiliszes the cytoplasm of the dying cells, hence preventing leakage of cytoplasmic contents into the extracellular space, an event which could lead to an immune response. Immunological characterization of the cross-linked matrix in apoptotic cells indicated the presence of actin, fibronectin, vinculin, annexin II, and other as yet uncharacterized proteins (Knight et al 1993). As well, a rearrangement of the actin microfilament network occurs in the cytoplasm of apoptotic cells to allow for cellular condensation and fragmentation in the formation of apoptotic bodies (Hale et al 1996; Martin and Cotter 1990; Cotter et al 1992).

In healthy cells, the anionic membrane lipid phosphatidylserine (PS), located in the inner plasma membrane, is inaccesible from the extracellular space, while sphingomyelin, phosphatidylcholine (PC), and the neutral phospholipids are found on the outer plasma

membrane of the bilayer (Fadok et al 1992). During apoptosis, PS is exposed on the outer membrane surface. For this reason, apoptotic but not healthy cells acquire the ability to recognize annexin V, a protein with a high affinity for PS (Homburg et al 1995). It has been proposed that the appearance of PS in the outer membrane is important for phagocytosis of apoptotic cells by neighbouring cells or macrophages (Fadok et al 1992).

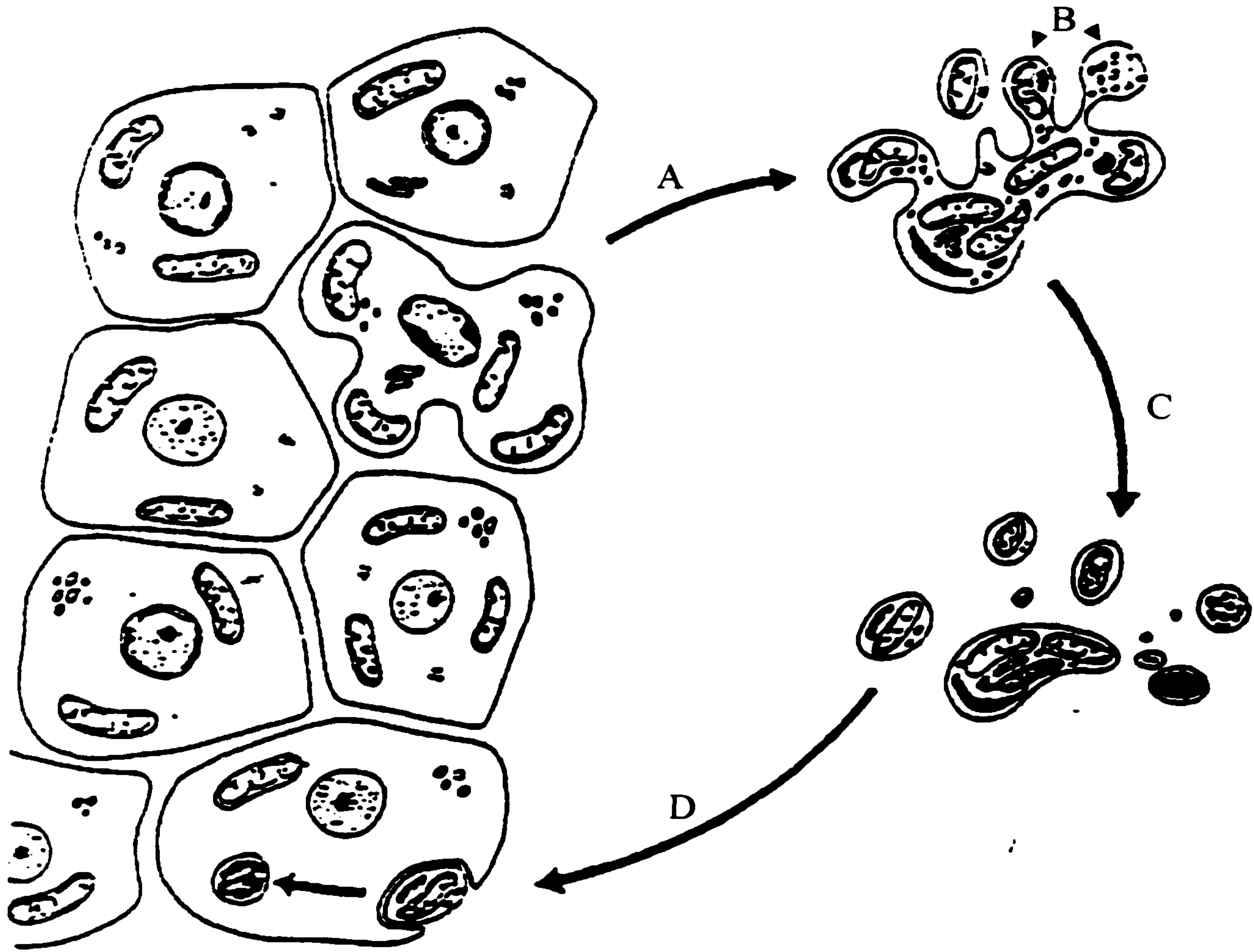
Although the mechanisms involved in inducing the nuclear, cytoplasmic and membrane events are largely unclear and not well understood, it appears that they can proceed in parallel (Jacobson et al 1994). Furthermore, the cytoplasmic and nuclear events can occur independently during apoptosis. For example, DNA fragmentation is not required for the cytoplasmic changes to occur (Jacobson et al 1994).

8.7.4 Molecular Aspects of Apoptosis

There is growing evidence suggesting that the process of apoptosis is very highly conserved throughout evolution, and that the diverse signals inducing or suppressing apoptosis eventually converge on limited number of evolutionary conserved genes. Bcl-2 and interleukin-1β-converting enzyme (ICE) proteins are members of two gene families involved with apoptosis. ICE members are cysteine proteases that cleave peptides/proteins at sites after aspartic acid. ICE members are now called 'caspases' (cysteine aspartase), and 13 caspases have been identified to date (Thornberry and Lazebnik, 1998; Humke et al 1998). Human homologues to caspases -11 and -12 have not been identified. All caspases exist in the cell in inactive forms (zymogens) which must be proteolytically activated to be functional. Caspases operate in a cascade where upstream caspases activate downstream caspases by proteolytic cleavage, in addition to cleaving their own substrate to destroy or activate it. Over-expression of the caspases results in apoptosis (Thornberry and Lazebnik, 1998). Caspase -1, -4, -5, -11, -12 and -13 are primarily involved in procytokine activation while other caspases are considered to

Figure 2. The morphological features of apoptosis

A: First, the apoptotic cell undergoes cytoplasmic condensation and pulls away from its neighbouring cells. B: Blebs (arrow head) are then formed at the cell membrane, as the cell prepares to fragment. The nucleus also disintegrates in the cells and chromatin is found in discrete dense fragments. C: The cells begins to fragment into membrane-bound vesicles called apoptotic bodies. D: The apoptotic bodies are quickly removed by neighbouring cells via phagocytosis, and digested in lysosomes.



modified from Kerr et al 1972

promote apoptosis. In addition to cleaving other caspases, caspases active in the apoptosis process degrade proteins required for cell viability and function, including poly (ADP)-ribose polymerase (PARP; an enzyme involved in DNA repair), DNA-dependent protein kinase (DNA-PK), sterol regulatory element binding proteins (SREBPs), small nuclear ribonucleoproteins (important in spliceosome assembly and mRNA processing; e.g. U1-snr), focal adhesion kinase (FAK; protein involved in maintaing cell adhesion), mdm-2 (protein involved in degrading p53) and Bcl-XL. (Nicholson and Thornberry 1997; Nagata 1997). Caspases also degrade proteins required for cell structure and support such as lamin, actin, and structual proteins in the nuclear scaffold (Thornberry and Lazebnik, 1998). Table 2 contains a list of the 13 known caspases and their substrates reported to date. The caspases implicated in apoptosis are currently divided into initiators and effectors. Generally caspase -2, -8, -9 and -10 are upstream components of the proteolytic cascade. Caspases -2, -3, -6 and -7 are involved in the effector phase of apoptosis.

Whereas the caspases work to induce or facilitate the apoptotic process, the bcl-2 proto-oncogene family members can both suppress and promote apoptosis. There are at least 12 mammalian members in the bcl-2 gene family that can form homodimers and heterodimers with one another. *Bcl-2, bcl-xlong, mcl-1* and *A1* inhibit apoptosis, whereas *bcl-xshort, bax, bad, bik, bid, bak, Harakiri, bim* and *bok* promote apoptosis (Adams and Cory, 1998). Consequently, each monomer can either enhance or suppress the function of the other dimers of the *bcl-2* family. In this way, the ratio of inhibitors to activators may determine the cell's propensity to undergo apoptosis. Overexpression of *bcl-2* or *bcl-xlong* blocks apoptosis induced by various triggers such as serum- or survival factor deprivation, irradiation, c-myc, Fas ligand (in few cell types), TNF-α and anti-cancer drugs (Boise et al 1995; Scaffidi et al 1998).

Table 2 Members of the caspase family and their known substrates

Protease	Substrate
Caspase-1 (ICE)	pro-interleukin 1β, pro-caspase 3 and 4
Caspase-2	PARP
Caspase-3 (CPP32)	Actin, PARP, DNA-PK, SREBP, rho-GDI, U1,DFF, MDM-2, BCL-XL, FAK, MEKK1, STAT
Caspase-4	?
Caspase-5	?
Caspase-6	lamin A
Caspase-7	PARP, pro-caspase 6 ,SREBP
Caspase-8 (FLICE)	Pro-caspase –3,-8,-10 and –13, Bid
Caspase-9	pro-caspase-3, PARP
Caspase-10 (FLICE2)	Pro-caspase-3
Caspase-11	pro-caspase-1 (no known human homologue)
Caspase-12	? (no known human homologue)
Caspaser-13 (ERICE)	?

? implies the substrate for that caspase is yet unknown
Thornberry and Lazebnik, 1998
Nicholson and Thornberry 1997

Bcl-2 and *bcl-xlong* are intergral membrane proteins that share similarity with the pore-forming domains of certain bactieral toxins (eg. diphtheria toxin). They are localized mostly in the outer mitochondrial membrane but with smaller amounts present in the endoplasmic reticular and nuclear membranes. *Bcl-2* family proteins have a profound influence on mitochondrial alterations associated with apoptosis. These include the loss of the electrochemical gradient across the inner membrane, generation of superoxide free radicals, release of matrix associated Ca2+ and the release of cytochrome c (cyt c) into the cytosol (Reed et al 1997). The anti-apoptotic members prevent these changes in the mitochondria whereas the pro-apoptotic members promote these changes.

It is postulated that these proteins can form channels that may transport proteins and/or ions. It is unclear how the anti-apoptotic members (which can also form ion channels) of the *bcl-2* family antagonize the effects of the pro-apoptotic members (Reed et al 1997) but it has been shown that Bax can directly induce release of cyt c from mitochondria (Jürgensmeler et al 1998). Caspase-8, activated by association with the intracellular domain of an activated TNF-α or Fas antigen receptor, can cleave the pro-apoptotic *bid* protein (Muzio et al 1996; Boldin et al 1996; Gross et al 1999). Once cleaved the fragment of *bid* translocates to the mitochondria and causes the release of cyt c. Apoptotic protease activating factor-1 (Apaf-1) is a recently cloned protein that is the mammalian homologue to CED-4 in *C. elegans* (Zou et al 1997). There is recent evidence that caspase function may be modulated by physical interaction wth Apaf-1, cyt c and *Bcl-2* family members (Pan et al 1998). Caspase-9 forms a multiprotein complex containing Apaf-1 and cytc which is dATP dependent (Li et al 1997). It has been proposed that cyt c initiates apoptosis by inducing the formation of the caspase-9/Apafl complex. Caspase-9 can then cleave caspase-3 (Hu et al 1998). The anti-apoptotic protein *Bcl-xl* can interact with caspase-9 and Apaf-1 resulting in the suppression of caspase-9 activation and therefore inhibited the activation of caspase-3. The association

of caspase-9 with pro-apoptotic and anti-apoptotic proteins suggests a major role for this protease in the control of apoptosis *in vivo*.

Activation of cysteine protease in this pathway depends on the nature of dimerization of *bcl-2* family members. Evidence suggests that certain cell survival factors suppress apoptosis by preventing apoptosis-promoting members from forming heterodimers with suppressor members of bcl-2. In the presence of survival factors, *bad* (a pro-apoptotic member of bcl-2) is phosphorylated on serine residues, presumably through AKT kinase (Wang et al 1996; Datta et al 1998). Phosphorylated bad loses its affinity for bcl-2 and is sequestered by the 14-3-3 protein.

In addition to caspases, it is becoming increasingly clear that signal transduction pathways involving specific protein kinases are involved in mediating apoptosis. Sustained activation of the JNK or p38 kinase pathways have been implicated in apoptosis (Yang et al 1997; Xia et al 1995). However, a number of reports have challenged the notion that the activation of JNKs and/or p38 kinases is sufficient to induce apoptosis (Smith et al 1997; Liu et al 1996). Thus, it appears that other signal pathways are required for apoptosis. However, the integration and balance of the JNK and p38 pathways probably do contribute to commitment to apoptosis.

Caspases can be regulated by the presence of inhibitors The family of IAPs (inhibitor of apoptosis proteins) can inhibit apoptosis in most cells. One family member, x-linked IAP has been shown to selectively inhibit caspase-3, -7 and pro-caspase-9 *in vitro* (Deveraux et al 1997, Deveraux et al 1998). IAPs can also inhibit apoptosis through non-caspase mechanisms, chiefly involving NF-κB. NF-κB can induce IAP expression and IAPs can in turn activate NF-κB (Stehlik et al 1998). IAPs block a wide spectrum of non-related apoptotic triggers, more so than *bcl-2* members, reflecting a site of action further downstream of *bcl-2*.

8.7.5 Apoptosis in Cancer

Dysregulaton of apoptosis, involving either inappropriate cell loss or failure to execute the apoptotic cascade, is the basis of many disease pathologies. Cancer and many of the autoimmune disorders can be conceptualized as the failure to execute apoptosis in cells which are destructive to the organism. Complete transformation of a cell may require both increased proliferative capacity and protection from apoptosis. The first clue that regulation of apoptosis influenced tumorigenesis came from studies of insulin-like growth factor 2 (IGF-2) in mice overexpressing the SV40 T-antigen (Tag). The IGF-2 null mice exhibited the same mitotic index as wild-type mice but there was an increase in apoptotic index and a decrease in tumor size. This suggested a role of enhanced survival rather than a mitogenic function for IGF-2, and a potential role of apoptosis in the regulation of tumorigenesis (Christofori et al 1994). Similar studies using transgenic mice expressing Tag and over-expressing bcl-xL demonstrate an increased frequency of tumor formation. However, early steps in multistep tumorigenesis are unaffected by the down-regulation of apoptosis afforded by bcl-2 overexpression (Naik et al 1996). Overexpression of XIAP and Hiap-2 was found in various cancer cells lines and a novel IAP termed survivin (Ambrosini et al 1997) was found to be expressed in cancers of many types as well as lymophoma, whereas it was almost absent in normal adult tissues. Taken together, these data suggest that a reduction of apoptosis, although not affecting the preneoplastic stages, may significantly enhance tumor progression when coupled with enhanced proliferation.

8.7.6 Apoptosis and CDDP Resistance

It has been postulated that a defect in the ability of the cells to undergo CDDP-induced apoptosis could contribute to CDDP resistance. Several components of the

apoptotic pathway have been examined in CDDP resistant cancer cells. As mentioned, the p53 protein plays a critical role in inducing apoptosis in response to CDDP-damaged DNA. It has been observed that the loss of p53 can significantly decrease the sensitivity of human ovarian cancer cells as well as other tumor cell types to CDDP (McCurrach et al 1997; Eliopoulos et al 1995). For example, the experimental development of CDDP resistance in vitro in IGROV-1 ovarian cancer cell lines is accompained by the development of p53 mutations and abrogation of the normal response to DNA damage, including upregulation of p53 and related downstream genes (Perego et al 1996). Transfection of the A2780-s cell line with a temperature sensitive p53 mutant induced resistance to CDDP (Eliopoulos et al 1995). Interestingly, the BRCA1 protein can interact with p53 *in vitro* and *in vivo* and can enhance p53-dependent gene expression by acting as a p53 coactivator (Ouchi et al 1998). It was recently found that the BRCA1 protein is overexpressed in CDDP resistant ovarian cancer cells exhibiting increased DNA damage repair. BRCA1 antisense expression resulted in increased sensitivity to CDDP, decreased proficiency of DNA repair, and enhanced rate of apoptosis (Husain et al 1998). Chemotherapeutic agents may induce apoptosis in the absence of a functional p53 and there is evidence that CDDP resistance may develop independently of p53 alterations (Coukos et al 1998).

It is possible that other factors controlling apoptosis may be overexpressed or deregulated in cancer cells. The *Bcl-2* protein, a negative regulator of apoptosis, has been found to be overexpressed in cancer cells. Transfection of *Bcl-2* or *Bcl-xL* can prevent CDDP-induced apoptosis in many cancer cells lines, including ovarian cancer (Eliopoulos et at 1995; Weller et al 1995). Some studies have shown that addition of general caspase inhibitors to ovarian cancer, human glioma, neuroblastoma and leukemia cell cultures can block apoptosis induced by CDDP, DXR and etoposide (Fulda et al 1998; Fuchs et al 1997). These studies suggest that since caspases seem to be necessary for CDDP induced apoptosis, their deregulation can result in CDDP resistance. Although

there has not been any demonstration of mutations in the caspases in drug resistant tumours and cell lines, it is conceivable that gene mutations pertaining to caspases or proteins involved in their activation could lead to the development of resistance to CDDP as well as other chemotherapeutic agents.

8.8 The Fas and Fas Ligand System

8.8.1 Fas

Death cell surface receptors that transmit apoptosis signals initiated by specific death ligands play a central role in apoptosis. Fas is a member of the TNF receptor superfamily which is defined by similar, cysteine-rich extracellular domains. Other members of this family include TNFR1 (p55 or CD120a), CAR1, death receptor 3 (DR3, Apo3, WSL-1, TRAMP, or LARD), DR4, DR5 (Apo2, TRAIL-R2, TRICK 2 or KILLER) (Ashkenazi and Dixit, 1998). The Fas receptor contains a cytoplasmic sequence termed the death domain (DD) that is common to the death receptors This domain enables the receptor to engage the cell's apoptotic machinery (Nagata and Golstein, 1995). The ligand that activates the Fas receptor is termed Fas ligand (FasL) and is a type II membrane protein that belongs to the TNF gene superfamily. Other members of this family include TNF and lymphotoxin α which binds to TNFR1; Apo3 ligand (TWEAK) which binds to DR3; Apo2 (TRAIL) which binds to DR4 and DR5 (Ashkenazi and Dixit, 1998). Fas and FasL play an important role in three types of physiologic apoptosis (i) peripheral deletion of activated mature T cells at the end of an immune response; (ii) killing of targets such as virus-infected cells or cancer cells by cytotoxic T cells and by natural killer cells; and (iii) killing of inflammatory cells at "immune-privileged" sites such as the eye (Ashkenazi and Dixit, 1998). Fas was discovered by two groups in 1989 after mouse derived antibodies were cytolytic for various human cell lines (Yonehara et al 1989; Trauth et al 1989). The gene for Fas is on chromosome 10, its mRNA is 12 kb and the protein is approximately 45 kDa. Many

tissues and cell lines weakly express Fas, but abundant expression was found in the thymus, liver, heart, lung, kidney and ovary (Nagata and Golstein 1995; Kim et al 1998).

Trimerization of the receptor results in aggregation of its intracellular DD, leading to the recruitment of a set of signaling proteins and the formation of the death-inducing signalling complex (DISC; Peter et al 1996; Figure 3). The Fas-associated death domain protein (FADD) is an adapter protein and is recruited to the Fas receptor and couples to the cross-linked receptor through its C-terminal DD. The N-terminal death effector domain (DED) enables the recruitment of FADD interleukin-1 β-converting enzyme (FLICE) or, as now commonly called, caspase-8 (Muzio et al 1996; Boldin et al 1996; Fernandes-Alnemri et al 1996). Physiological caspase-8 cleavage requires association with the DISC and occurs by a two step-mechanism. Initial cleavage generates a p43/p41 and a p12 fragment and the p12 is further processed to a p10 fragment. Subsequent cleavage of the p43/p41 fragment results in the formation of a p26 fragment and release of the active site containing fragment p18. The p43/p41 and p26 fragments contain the two DED domains (Medema et al 1998). There they can activate other caspases, including caspase-3, in turn resulting in the specific cleavage of a number of "death substrates" (Scaffidi et al 1998). Activated caspase-8 can cleave the *bid* protein into a p15 fragment which translocates to the mitochondria and causes the release of cyto c (Gross et al 1999). The Fas signalling pathway downstream of the DISC, has been shown to involve the activation of additional caspases such as caspase-1, -3, -4, -6, -7 (Nagata 1997) -10 (Vincenz and Dixit 1997) and -13 (Humke et al 1998). In mice deficient in caspase-3, the Fas apoptotic pathway was not affected in most tissues suggesting that the participation of other effector caspases is sufficient (Kuida et al 1996). The viral inhibitors, the molluscum contagiosum virus protein MC159 and the equine herpesvirus protein E8, encode DED-containing decoy molecules that bind to either FADD (MC159) or caspase-8 (E8) and disrupt assembly of the receptor signaling complex, thereby abrogating the death signal. A mammalian inhibitor designated FLAME1 (FLIP/I-

FLICE), containing two N-terminal DED's but lacking a catalytic active site, binds to caspase-8 and 10 and blocks their association with FADD (Hu et al 1997).

Fas signaling may proceed by two pathways, one involves mitochondrial factors and one in which the mitochondria plays a minimal role. The pathway that is used once Fas is activated seems to depend on the cell type. Type I cells (e.g B lymphoblastoid and T-cells) use the mitochondria-independent apoptosis pathway and are not inhibited by *Bcl-xL*. Type II cells (Jurkat cells) are mitochondria dependent (Scaffidi et al 1998). It has been suggested that in the presence of caspase-3 and the levels of active caspase-8 at the DISC will determine whether the apoptotic pathway will be mitochondria-dependent or not.

In addition, a signaling protein Daxx can bind to the Fas DD and induce apoptosis as well as the Jun N-terminal kinase (JNK) pathway (Yang et al 1997). This pathway acts cooperatively with FADD and is Bcl-2 inhibitable. Activation of Fas may also result in generation of ceramide, the role of which in apoptosis is unclear (Cifone et al 1994; Hsu et al 1998). Several kinases (tyrosine and serine/threonine kinases) have also been shown to be associated with Fas and may modulate the main death pathway initiated by Fas in a cell-specific manner (Peter et al 1996). In some cell lines, anti-Fas antibodies do not trigger apoptosis, but induce activation of the transcription factor NF-κB and/or interleukin-8 secretion (Abreu-Martin et al 1995).

8.8.2 Fas Ligand (Fas L)

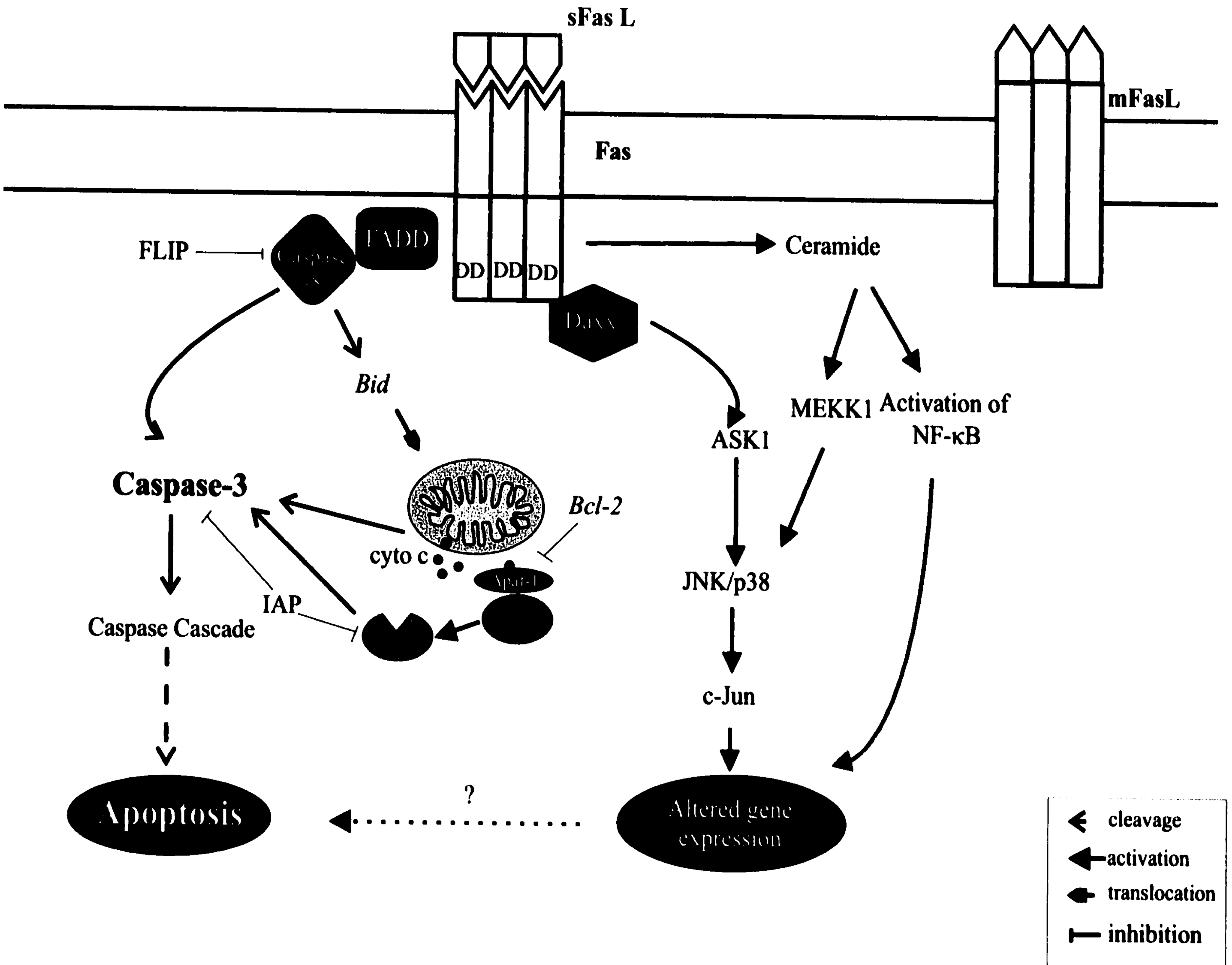
FasL is a 40 kDa type II membrane protein belonging to the TNF family. The human FasL gene consists of 8.0 kb and maps to chromosome 1. The region upstream of the ATG start codon contains several transcription elements such as SP-1, NF-κB and IRF-1 (Takahashi et al 1994). The FasL is modified by N-Linked glycosylation, which is not required for biological activity. The FasL receptor binding site is found on the

extracellular domain (Orlinick et al 1997). FasL is predominantly expressed in activated T cells and natural killer (NK) cells. Analyses of mice lacking Fas or FasL have indicated that FasL is one of the major effector molecules of cytotoxic T lymphocytes (CTL). However, when this system overfunctions, it causes tissue destruction. The involvment of FasL-induced apoptosis in CTL-mediated autoimmune diseases (e.g Hashimotos disease) has been demonstrated (Giordano et al 1997). FasL expression also contributes to immune privelege (eye, testis and brain) by protecting transplants from infiltration and destruction by activated T cells and NK cells (Nagata 1996).

Membrane-bound FasL can be cleaved to become a 26 kDa soluble form (sFasL). Since metalloproteinase inhibitors prevent the shedding of FasL, it has been suggested that a metalloproteinase is responsible for the production of sFasL. Membrane-bound form of FasL is cleaved between Lys 129 and Gln 130 to create a soluble form (Tanaka et al 1998). The human FasL is functional in inducing apotposis in activated lymphocytes (Tanaka et al 1995) and cell lines that produce abundant levels of Fas (Tanaka et al 1998). However, Tanaka et al (1998) demonstrate that cells expressing low levels of Fas were resistant to sFasL and suggest that sFasL may actually act to downregulate the cytotoxic activity of FasL. Soluble FasL has been detected in the serum of human patients suffering from large granular leukemia as well as NK lymphoma (Tanaka et al 1996). To exhibit a lethal effect in mice, large amounts of sFasL were needed, suggesting that their cytotoxic activity was less than that of membrane bound FasL (Tanaka et al 1997). Fas-sFasL complexes are rapidly internalized, and this may prevent formation of the DISC. Signaling may instead occur through Daxx, which triggers apoptosis more slowly and less efficiently, possibly through JNK and activation of as-yet unknown caspases (Yang et al 1997).

Figure 3. Fas and Fas ligand signaling system.

Trimerization of the Fas receptor occurs upon ligand binding and reruits the adapter FADD. FADD then recruits caspase-8, which upon activation can cleave caspase-3 directly and/or indirectly through *Bid* cleavage and activation of mitochondrial factors. Caspase-3 then cleaves substrates necessary for the execution of apoptosis. Daxx can interact with the DD of the trimerized Fas receptor and result in JNK activation, leading to altered gene expression and presumably cell death.


sFas L
mFasL
Fas
FLIP
FADD
DD DD DD
Daxx
Ceramide
Bid
MEKK1
Activation of NF-κB
ASK1
Caspase-3
Bcl-2
cyto c
Apaf-1
JNK/p38
IAP
Caspase Cascade
c-Jun
?
Apoptosis
Altered gene expression
cleavage
activation
translocation
inhibition

8.8.3 The Fas and FasL system and cancer

Since activation of the Fas/FasL system induces apoptosis, dysregulation of this system may confer survival onto cells and may contribute to tumorigenesis. The loss of Fas protein has been reported to induce resistance to apoptosis (Keane et al 1996). However, apoptotic resistance in some Fas-expressing malignant cells has also been reported (Owen-Schaub et al 1994). Abnormalties in Fas protein can occur due to mutations of the Fas gene or abnormalities in post-translational processing of Fas (Nambu et al 1998; Tamiya et al 1998). Loss of Fas expression has been demonstrated in hematopoietic malignancies, colon, testicular, hepatocellular and breast cancer as well as pulmonary adenocarcinomas (Nambu et al 1998; Keane et al 1996). Biochemical data also support a role for Fas in tumorigenesis. Fas induced apoptosis is impaired in hepatocytes from transgenic mice expressing SV40T antigen in the liver (these mice develop hepatocellular carcinoma) and Fas sensitivity can be restored to colon cancer cells by expression of wild-type p53 (Rouquet et al 1995).

Fas protein can occur as both a cell-surface and a soluble protein, with four isoforms of the soluble form of Fas (sFas) being generated by alternative mRNA splicing (Cheng et al 1994; Cascino et al 1995). This sFas can protect the cell against Fas-mediated apoptosis by neutralizing the FasL (Cheng et al 1994). Elevated sFas levels in sera from patients with non-hematopoietic human malignancies has been reported, with elevated expression of sFas protein in tumor cells causing apoptotic resistance (Owen-Schaub et al 1994). Taken together, these data support a role for the loss of Fas mediated apoptosis during tumorigenesis and/or tumor progression.

FasL expression has to be tightly controlled to prevent destruction of tissue sensitive towards Fas-mediated apoptosis. Cells with downregulated Fas, but expressing FasL, can counter-attack Fas expressing lymphocytes. T-lymphocytes which would

normally kill the tumor cells are then eliminated through Fas-mediated apoptosis (Nagata 1996). The FasL expressing tumor thus behaves like an immune privileged site. Expression of FasL by tumors cells and the killing of infiltrating Fas-expressing activated cytotoxic T lymphocytes was found to occur in a melanoma. In addition, FasL and the sFasL have been detected in tumor cells of melanoma lesions (Hahne et al 1996). Immune escape with the help of FasL has been established for colon cancer cell lines, as well as for primary and metastatic colon tumors (O'Connell et al 1996).

Fas and FasL expression has been detected in the ovary. Monoclonal antibodies to Fas induce DNA fragmentation in cultured human granulosa cells (Quirk et al 1995). Injection of mice with anti-Fas antibodies also induced apoptosis in both the corpus luteum and follicles, indicating a role for Fas/FasL system in luteal regression and follicular atresia (Sakamaki et al 1997). In the adult human ovary, Fas expression has been demonstrated in the oocyte and decreased with advance in follicular maturation (Kondo et al 1996). Fas and FasL expression and function in human OSE cells have not been determined. They may play a role in the induction of apoptosis in the OSE cells during ovulation and their dysregulation may contribute to ovarian cancer.

9.0 HYPOTHESIS

CDDP induces apoptosis in ovarian cancer cells by increasing the expression of Fas and/or FasL. The loss of responsiveness of ovarian cancer cells to this agent may be a result of its inability to up-regulate Fas and/or FasL expression and to activate the Fas apoptotic pathway.

10.0 OBJECTIVES

I To examine the susceptibility of ovarian cancer cells to CDDP in the induction of apoptosis.

II To determine if the Fas signaling pathway is involved in CDDP cytotoxicity.

III To examine the involvment of the Fas and FasL system in CDDP resistance.

11.0 SPECIFIC AIMS

A. Ability of human ovarian cancer cells to undergo apoptosis

1. To determine if two pairs of CDDP-sensitive and -resistant hOSE cancer cells will undergo apoptosis upon serum withdrawal.

2. To determine the responsiveness of hOSE cancer cells to CDDP in terms of Fas-induced apoptosis.

B Expression and regulation of Fas, FasL and sFas in hOSE cancer cells by CDDP and their role in chemoresistance

1. To determine the presence of Fas and FasL protein in human ovarian carcinoma.

2. To examine the expression of Fas and FasL protein in two pairs of CDDP-sensitive and -resistant hOSE cancer cell lines *in vitro*.

3. To compare the expression of Fas, FasL and sFas in two pairs of CDDP-sensitive and -resistant hOSE cancer cell lines in response to CDDP *in vitro*.

4. To determine if Fas is essential for CDDP-induced apoptosis in a CDDP-sensitive cell line.

C. The involvment of Caspase-3 and -8 in CDDP-induced apoptosis.

1. To determine if CDDP-induced apoptosis involves the activation of caspase-3 and -8 in hOSE cancer cells.

D. Functionality of Fas and FasL in hOSE cancer cells

1. To determine whether CDDP can enhance Agonistic Fas monoclonal Ab induced cytotoxicity in hOSE cancer cells.

12.0 MATERIALS

QIAamp Blood Kit, QIAquick Nucleotide Removal Kit and ribonuclease A (RNase A) were purchased from Qiagen Inc. (Chatsworth CA, USA). *cis*-Diamminedichloroplatinum (II), Aprotinin, PMSF, ammonium persulfate, Tween 20, bis-benzimide trihydrochloride (Hoechst 33258), agarose, mouse IgG and IgM, ethidium bromide and Nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis Mo., USA). N,N-Dimethyl Formamide, sodium dodecyl sulphate, bromophenol blue, trizma base, sodium chloride, ethanol and formaldehyde were purchased from BDH (Toronto, Ontario, Canada). Culture media (RPMI1640 and DMEM/F12), culture media reagents (Non essential amino acids, penicillin, streptomycin, fungizone and trypsin-EDTA) and fetal bovine serum (FBS) were purchased from Gibco/Bethesda Research Laboratories (Burlington, Ontario, Canada). Human ovarian epithelial cancer cells (OV2008, C13*, A2780-s and A2780-cp) were gifts from Dr. Goal and Dr C.E. Ng respectively (Ottawa Regional Cancer Centre, Ottawa, Ontario, Canada). Glycine, acrylamide, N,N-methylene, bis-acrylamide, prestained low-range molecular weight markers, HRP linked goat anti-rabbit IgG, horse radish peroxidase (HRP) linked rabbit anti-mouse IgG and DC Protein Assay kit were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). TEMED, dithiothreital (DTT), Coomassie blue were obtained Boehringer Mannhein (Montreal, Quebec, Canada). Klenow polymerase was purchased from New England Biolabs (Beverly MA, USA). α-[^{32}P]-dCTP (250μCi/vial) and enhanced chemiluminescence (ECL) kit were obtained from Amersham Life Science (Oakville, Ontario, Canada). Anti-human mouse monoclonal Fas and FasL antibodies (Abs) were purchased from Transduction Laboratories (Mississauga, ON, Canada). Anti-human rabbit polyclonal Fas Ab was purchased from Santa Cruz Biotechnology (Santa Cruz,, CA, USA). Agonistic and antagonistic anti-human monoclonal Fas Abs were purchased

from Upstate Biotechnology (CH-11 and ZB4 respectively; Lake Placid, NY; Yonehara S et al 1989; Yonehara S et al 1994). Anti-human rabbit polyclonal caspase-3 Ab was purchased from Pharmingen (Mississauga, ON, Canada). Centricon spin columns were purchased from Amicon (Beverly, MA, USA). Monolconal human anti-caspase-8 Ab (C15) was a gift from Dr M. Peter (German Cancer Research Center, Heidelberg, Germany).

13.0 METHODS

13.1 Ovarian Cancer Cell Culture

CDDP-sensitive cells (OV2008 and A2780-s) and their resistant variants (C13* and A2780-cp, respectively) were cultured at 37°C and 5% CO_2 in either RPMI 1640 (OV2008, C13*) or DMEM-F12 (A2780-s, A2780-cp) (Gibco/BRL, Burlington, ON). Media was supplemented with 1% non-essential amino acids, 0.5% streptomycin-penicillin, 0.25% fungizone and 10% fetal bovine serum for cell plating. All treatments were performed on cells in the log growth phase in serum-free media for up to 24h. OV2008 and C13* hOSE cancer cells have previously been characterized by Andrews et al (1990) while A2780-s and A2780-cp by DiSaia et al (1972) and Masuda et al (1990), respectively.

13.2 Biochemical Assessment of Apoptosis (DNA Ladders)

13.2.1 DNA Extraction and Quantification

DNA was isolated using the QIAamp Blood Kit (Qiagen, Inc.) as per manufacturer's instructions. Briefly, cells were lysed with an acidic salt solution and subject to RNase and proteinase K treatment to digest RNA and protein. Samples were loaded onto spin columns containing silica membranes which specifically bound DNA (80 bp to 50 kb in size). Salt was removed from the columns by washing twice with an ethanolic solution, and finally DNA was eluted with tris buffer (10 mM, pH 9). Eluted DNA was stored at -80°C pending 3'-end labelling with α-[^{32}P]-dCTP (deoxycytidine 5'-α-[^{32}P] triphosphate). To quantify the DNA in the samples, 50µl of the above DNA eluate was added to 450 µl tris (10 mM, pH 9). The absorbance of this final solution was

measured in a quartz cuvette at 260 nm, using a Milton Roy Spectronic 1201 spectrometer. Fifty ng DNA/ μl gives an absorbance (OD) unit of one.

13.2.2 DNA 3' -end labelling

To quantify the extent of DNA fragmentation in cultured cells, the isolated DNA was radiolabelled at the 3' -end with α-[^{32}P]-dCTP, using the Klenow reaction as described by Rosl (1992) and resolved on 1.8 % agarose gel electrophoresis (AGE). In this reaction, the Klenow polymerase ligated the α-[^{32}P]-dCTP to the free 3' hydroxyl group (on the 5'→3' strand) when the nucleotide base on the complementary DNA strand was guanine. The reaction was allowed to proceed at RT for 30 minutes The labelled DNA was purified with the use of the Nucleotide Removal Kit (Qiagen Inc.) as per manufacturer's instructions. Briefly, this involved adsorption of the DNA onto a silica membrane in a spin column under low pH and high salt conditions, followed by washing of the column with an ethanolic solution to remove salt. DNA (labelled) was eluted with tris buffer (10 mM, pH 8.5) and stored at -20°C pending AGE.

13.2.3 Agarose Gel Electrophoresis and Autoradiography

Labelled DNA of different sizes was resolved by AGE according to the method of Sambrook et al (1989). Briefly, radiolabelled DNA was mixed with gel loading buffer (GLB; 0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol in water), loaded 25 μl/ well in agarose gel [1.8% in tris-acetate EDTA buffer [TAE; tris (4.84 g/L), glacial acetic acid (1.14 ml/ L), EDTA (0.001M)] and resolved at 60 volts for about 150 minutes. GLB provided a dense medium for reliable sample loading and contained a dye (bromophenol blue which migrated at the same rate as 300 bp double stranded DNA) to monitor the extent of electrophoresis. The gel was dried under negative pressure (no

heat) in a gel dryer (Drygels Sr Model SE 1160) for 4 hours and exposed to X-ray film for 2-24 hours. The X-ray film was then developed in Kodak M35A X-OMAT processors.

13.2.4 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL)

In situ cell death detection by the TUNEL technique was carried out as previously described (Gavrieli et al. 1992). Briefly, tumour sections were treated 37°C, 10 min with proteinase-K (10 μg/ml in 20 mM Tris; 2 mM $CaCl_2$, pH 7.4), washed with Tris buffer (100 mM Tris; 150 mM NaCl, pH 7.5; 15 min) and immersed in methanol containing 0.3 % H_2O_2 (RT, 20 min). After a 15 min rinse with distilled water, the sections were incubated in the terminal deoxynucleotidyl transferase (TdT) buffer (25 mM Tris-HCl, 200 mM sodium cacodylate, 5 mM cobalt chloride, 250 μg/ml bovine serum albumin, pH 6.6; 15 min) and then in 50 μl of TdT buffer containing 10 U TdT and 1 nmol biotinylated 16-dUTP in a humidified chamber (37 °C, 60 min). The biotinylated dUTP molecules incorporated into nuclear DNA were visualized following incubation with HRP-conjugated streptavidin (1:100; RT, 30 min) and DAB solution (5 min). In the negative control slides, TdT or biotinylated 16-dUTP were omitted in the labeling reactions.

13.3 Morphological Assessment of Apoptosis

13.3.1 Phase Contrast Microscopy

At the end of the culture period, cells were viewed under phase contrast microscopy, using an (Axiovert 35 inverted microscope; Carl Zeiss Inc. Germany) and photographed in bright field at high (400X) magnification.

13.3.2 Hoechst Staining of Cultured Cells

At the end of the culture period, the medium (containing floating cells) was transferred to a 1.5 ml microfuge tube. The cells were detached from the culture surface by incubating in trypsin -EDTA (0.025 g/ml in PBS; 37 °C; 2 min) and combined with floating cells in the medium fraction, and fixed with 37% formaldehyde (10% v/v; RT, 10 min). Next, cells were incubated with Hoechst 33258 dye (2 μg/ml in PBS; RT. 24h). Hoechst 33258 dye, which interacts with double stranded DNA and emits visible light when excited by UV, was used to assess nuclear morphology of ovarian cancer cells. An aliquot of the cell suspension was placed on a glass slide, viewed under UV light under a Zeiss IM 35 microscope (Carl Zeiss Inc. Germany) and photographed at 400X magnification. Cells with fragmented nuclei or highly condensed nuclei (which stain brightly) were considered apoptotic. Between 200-400 cells were counted in each treatment group in every experiment.

13.4 Protein Analysis

13.4.1 Protein Extraction and Quantification

All procedures were carried out on ice or under refrigeration (4°C). Floating and adherent cells were pooled and sonicated (1 min) in a lysis buffer (pH 7.4) containing 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 in PBS and protease inhibitors [PMSF (1 mM), aprotinin (10 μg/ml)]. The sonicates were then incubated for 1h and pelleted (30 min, 16,000 x g). Supernatant was retained and stored at -20°C, pending Western analysis. Spent culture media was processed as described by Tanaka et al (1995). Briefly, spent culture media was filtered through a 0.22 micron syringe filter (Millipore, Bedford, MA) to remove cell debris followed by centrifugation (30 min, 393, 201 x g) and the media was then passed through a Centricon spin column (molecular

weight cut-off of 10 kDa) and concentrated 40X. Protein content of the extracts was determined with the Bio-Rad DC Protein Assay.

13.4.2 Western Blot Analysis

Protein extracts (15-30 μg) were resolved by SDS-PAGE (Laemmli, 1970). Protein extracts were loaded onto standard Laemmeli gels (10%). The proteins on the gel were then electrotransferred (30V, 16h) onto nitrocellulose membranes which were then blocked (RT, 2h) with TBS-T containing 5% skim milk powder, and incubated (RT, 1h) with primary antibody diluted in TBS-T and 5% skim milk powder. The blots were then washed in TBS-T (3 X 5 min, incubated with HRP- conjugated secondary antibody (1:10,000) in TBS-T and 5% skim milk powder (RT, 1h) and washed again in TBS-T (3 X 5 min) and TBS (1 X 5min). Peroxidase activity was visualized with the ECL kit, as per manufacturer's instructions. Primary Ab dilutions were as follows: polycolonal anti-Fas Ab (1:4000), monclonal anti-Fas and anti-FasL Ab (1:2000), polyclonal anti-caspase-3 Ab (1:8000), monoclonal anti-caspase-8 (1:1000). Membranes were blocked in 2% BSA in PBS and 0.05% T20 instead of skim milk when using the anti-caspase-8 Ab. The gels were stained with Coomassie R250 after transfer to confirm equal protein loading. Densitometric analysis was performed using a Document Scanner and Molecular Analyst Software from Bio-Rad Laboratories (Mississauga, ON, Canada).

13.5 Soluble FasL Activity

OV2008 cells were cultured in serum-free media with or without CDDP (30μM) for 24h. The spent media was concentrated 48X to a volume of 500μl using a centricon spin column with the same molecular weight cut-off as mentioned previously. The concentrated spent media was then transferred to subconfluent cultures of C13* cells.

Agonist Fas mAb (2μg/ml) was added at the same time as the concentrated media. To determine if the cell death was due to the FasL in the media, cells were preincubated for 6h with an antagonistic anti-Fas Ab (10μg/ml) that blocks the Fas receptor.

13.6 Preparation of human ovarian epithelial carcinoma

Ovarian surface epithelial carcinomas were obtained as pathologic specimens from three patients (ages ranging from 57 to 76 years) who were undergoing their initial laparotomy for surgical staging and tumour debulking. None had previously received any chemotherapeutic agent and all three patients were found to have stage III disease at the time of surgery. The tumour tissues were washed with PBS (pH 7.4), and then immediately fixed in 10% neutral buffered formalin (pH 7.4) for 24h and followed by routine paraffin embedding procedure. Adjacent paraffin tissue sections (4-5 μm) were deparaffinized with xylene (2 x 5 min) and hydrated with a series of graded ethanol (50-90%) and subsequently with PBS for further histological analysis.

13.7 Immunohistochemistry

Immunohistochemical localization of Fas, FasL and PCNA in the tumour sections was carried out as described previously (Kim et al. 1998). Briefly, tumour sections were incubated in series with 0.3 % H_2O (20 min), PBS (3 x 15 min), 1.5 % normal goat serum (RT, 1h), and with rabbit polyclonal anti-human Fas or FasL (0.3 μg/ml), or mouse monoclonal anti-human PCNA (0.2 μg/ml) antibodies (in 1 % blocking serum; RT, 45 min). After three washes (15 min each) with PBS, the sections were then incubated with biotin-conjugated goat anti-rabbit IgG or anti-mouse IgG (1:200; RT, 1h), followed by avidin-biotin-peroxidase complex (RT, 1 h). They were washed with PBS (3 x 15min) and incubated with diaminobenzidine tetrahydrochloride (DAB) solution (0.3 mg/ml DAB, 0.05 M Tris-HCl buffer, pH 7.6, 0.003 % H_2O_2) for 2-5 min. The nuclei

were counterstained with hematoxylin. Rabbit or mouse IgG (1 μg/ml) was used instead of primary antibody for the negative control reaction.

13.8 Statistical Analysis

All data are expressed as mean ± SEM from 4-7 experiments. Studies were statistically analysed by analysis of variance. Significant differences between groups were determined by the Tukey's test. Statistical difference was inferred at $p<0.05$.

14.0 RESULTS

14.1 Serum Deprivation

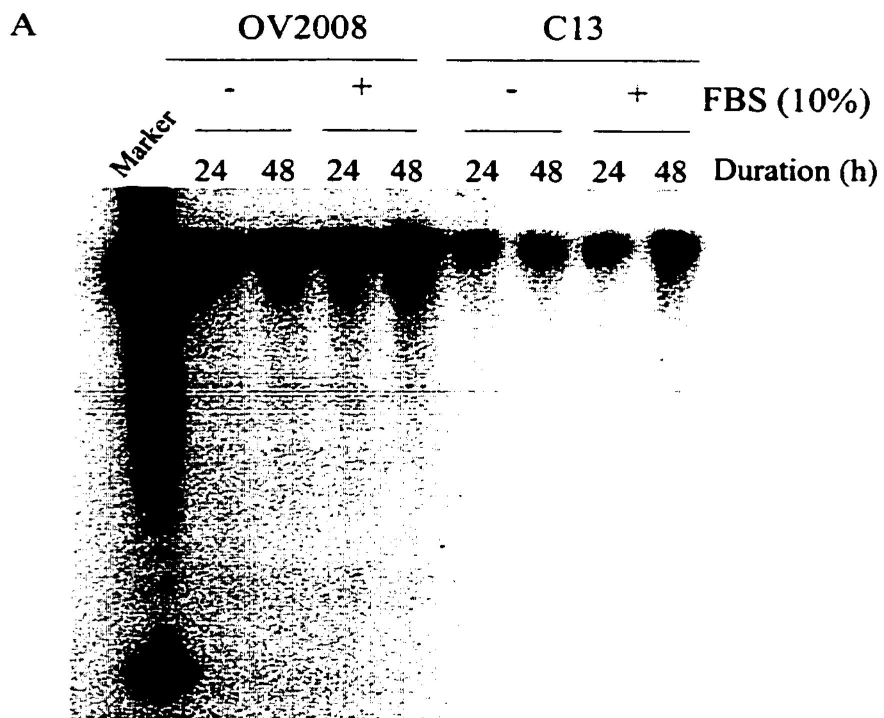
It has been reported in many cell types that serum-deprivation *in vitro* induces apoptosis (Raff, 1992). To determine whether the CDDP-resistant cells (C13*, A2780-cp) were inherently more resistant to the induction of apoptosis than the sensitive counterparts (OV2008, A2780-s, respectively), these ovarian cancer cells were subjected to serum-deprivation for up to 48h. Cells were plated for 24 h in the presence of 10% FBS, then further cultured for up to 48h in media with or without serum. DNA from the serum containing and deprived cancer cell cultures did not display DNA fragmentation (Fig 4 A and B). CDDP-resistant cells do not display an increased resistance to apoptosis in response to serum-deprivation. Since serum-free conditions failed to induce apoptosis in these cancer cells up to 48h, subsequent studies were carried out in serum-free conditions up to 24h, to obtain a more defined culture system. As a positive control rat granulosa cells were cultured in the presence and absence of serum for up to 24 h. DNA fragmentation was observed in the granulosa cells cultured under serum free conditions (Fig 4 C). LMW DNA fragmentation has previously been observed in OV2008 and A2780-s cells lines in response to apoptosis inducing agents (Liu et al 1994; Song et al 1997).

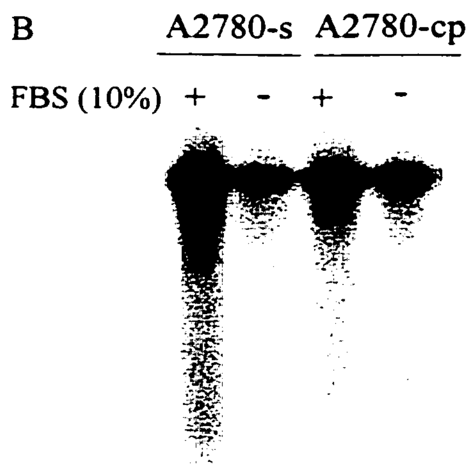
14.2 Morphologically Healthy vs Apoptotic hOSE cancer cells

CDDP-sensitive (OV2008) and -resistant (C13*) human ovarian epithelial cells cultured in the absence of chemotherapeutic agents exhibited normal cellular morphology as evident by their adherence to the growth surface and flat, cobblestone-like

Figure 4. Serum deprivation does not cause hOSE cancer cells to undergo apoptosis.

Ovarian cancer cells were plated for 24h in media containing 10% FBS, then cultured up to 48h with or without serum. DNA was isolated, 3' end-labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing the absence of DNA ladders in OV2008 and C13 Panel B: representative autoradiogram showing the absence of DNA ladders in A2780-s and A2780-cp. Panel C representative autoradiogram showing the presence of DNA ladders upon serum withdrawal in rat granulosa cells. N=3 experiments.


A
OV2008
C13
-
+
-
+
FBS (10%)
Marker
24
48
24
48
24
48
24
48
Duration (h)


B
A2780-s
A2780-cp
FBS (10%)
+
-
+
-

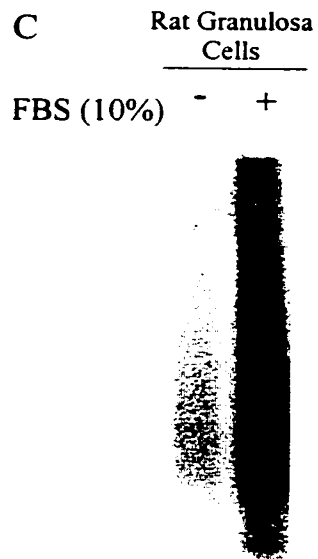
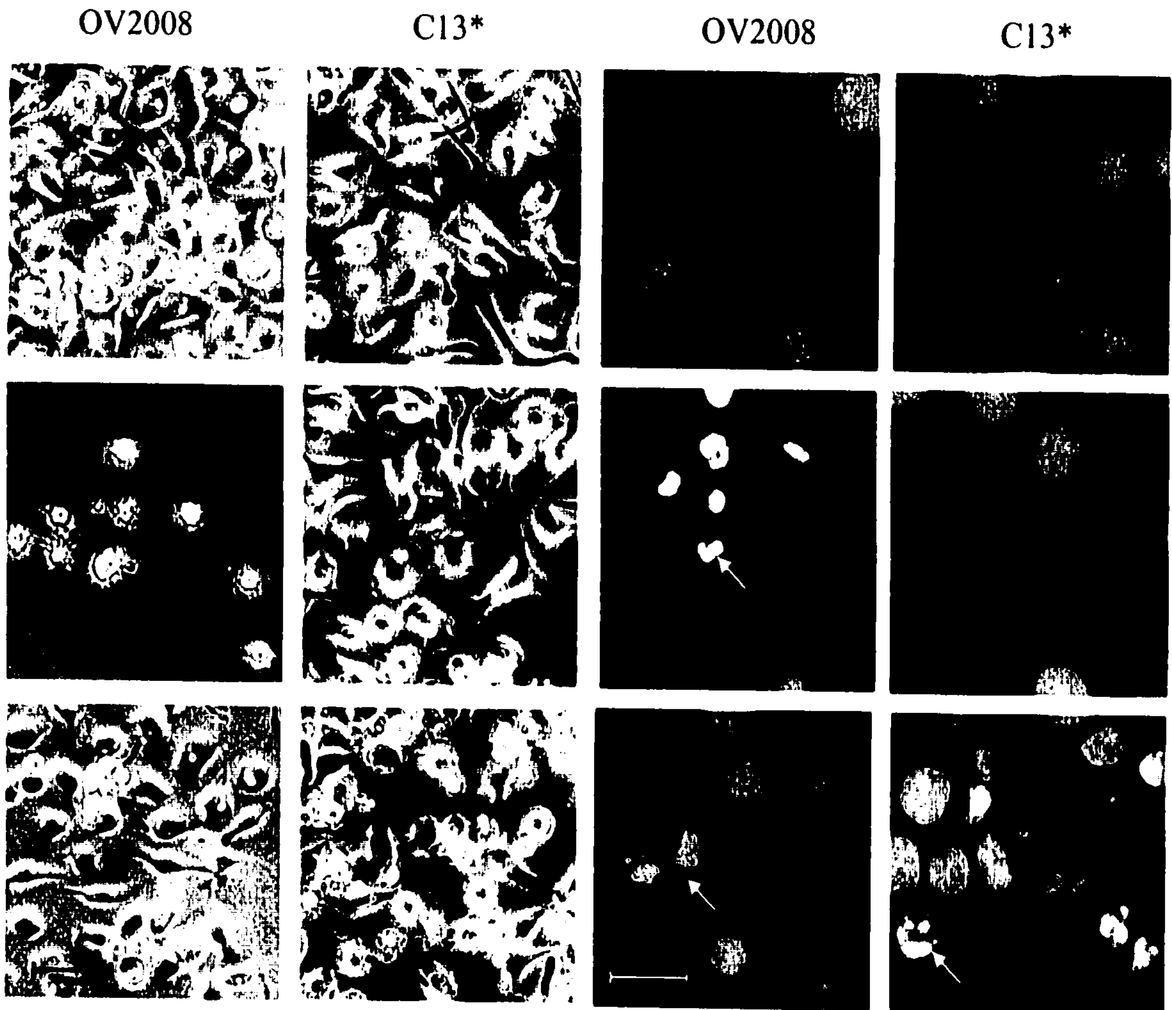

C
Rat Granulosa Cells
FBS (10%)
-
+

Figure 5. Phase contrast photomicrograph and assessment of nuclear morphology in OV2008 and C13* hOSE cancer cells.

Representative photomicorgraphs showing general (Panel A; phase-contrast microscopy) and nuclear (Panel B; Hoechst staining) morphology of CDDP-sensitive (OV2008) and -resistant (C13*) ovarian epithelial cancer cells after treatment for 24h with CDDP (30 μM) or an agonistic Fas mAb (2 μg/ml). Black arrows (Panel A) shows cellular blebbing and detachment and white arrows (Panel B) indicate nuclear fragmentation. Magnification: 400X.


A
B
OV2008
C13*
OV2008
C13*
Control
CDDP
Fas mAb

appearance (Fig. 5A). Addition of CDDP (30 μM) or an agonistic monoclonal anti-Fas antibody

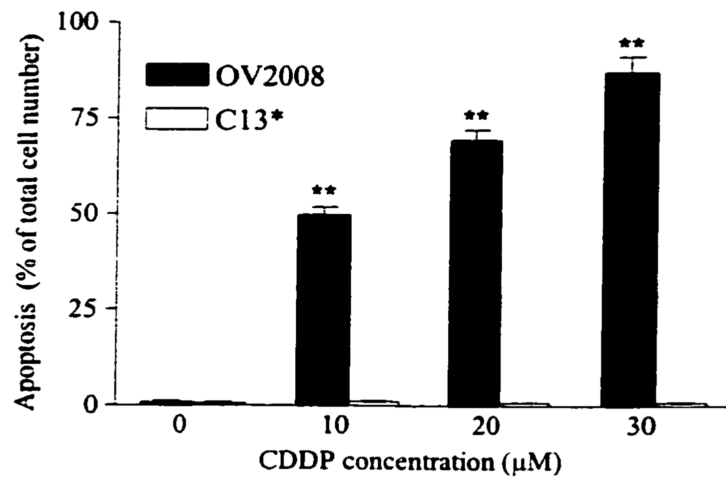
(Fas mAb; 2 μg/ml), induced apoptotic features in OV2008 cells [e.g. cell rounding and shrinkage, formation of apoptotic bodies (Fig. 5A), nuclear condensation and nuclear fragmention (Fig. 5B)]. In contrast, C13* cells did not undergo cellular or nuclear morphological changes indicative of apoptosis in response to CDDP (Fig. 5A and B). As observed with OV2008, Fas activation with Fas mAb in its resistant variant resulted in typical apoptotic cellular and nuclear morphology, although these changes were somewhat less extensive as those observed in CDDP-treated OV2008 cells (Fig. 5A and B). The effects of CDDP on apoptosis in OV2008 was concentration- and time-dependent ($p<0.01$). Whereas C13* cells failed to respond to CDDP at concentrations as high as 30μM, the LD50 of this chemotherapeutic agent in the sensitive cells was about 10μM (Fig. 6A). In addition, whereas as much as 85% of the sensitive cells were apoptotic in a 24h culture with CDDP (30μM), minimal cell death (<2%) was evident in the resistant cells under identical conditions (Fig. 6B). These studies confirm earlier findings that CDDP induces cell death in ovarian cancer cells through apoptosis and suggests that Fas and its postreceptor events are functional but suboptimal in both CDDP-sensitive and -resistant cells.

High concentrations of CDDP can induce necrosis in tissue culture systems (Lieberthel et al 1996; Guchelaar et al 1998). Cell swelling and bursting are features of necrosis and will lead to cell loss (Lieberthel et al 1996). Since the assessement of apoptosis used in this study is based on cell number OV2008 cells were counted after CDDP treatment to determine if there was a significant loss of cells due to the treatment. CDDP did not significantly affect the cell number until a concentration of 30 μM (Fig. 7A). At this concentration, the cell number significantly dropped by 20 % ($p<0.05$; Fig.7B). Treatment of OV2008 with agonist Fas Ab did not significantly affect cell number after 24h (Fig. 7B).

Figure 6. Concentration- and time-dependent apoptosis after CDDP treatment.

CDDP-induced apoptosis in CDDP-sensitive (OV2008) but not -resistant (C13*) human ovarian epithelial cancer cells. Panel A, concentration-response study during a 24h culture period in the presence of various concentrations (0-30μM) of CDDP. Panel B, time-course study on the incidence of apoptosis in OV2008 (______) and C13* (_ _ _ _ _) cultured in the absence (❍) and presence (●) of CDDP (30μM) for a 24h period. Nuclear morphology was detected by Hoechst stain and those cells displaying nuclear condensation and fragmentation were counted as apoptotic. Data represent mean ± SEM of three (Panel B) or four (Panel A) experiments. $^*p<0.05$, $^{**}p<0.01$ (compared to respective control).

A



B

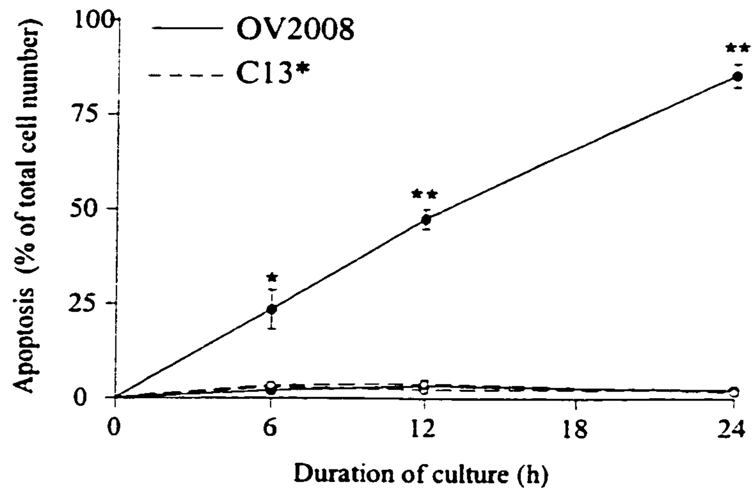
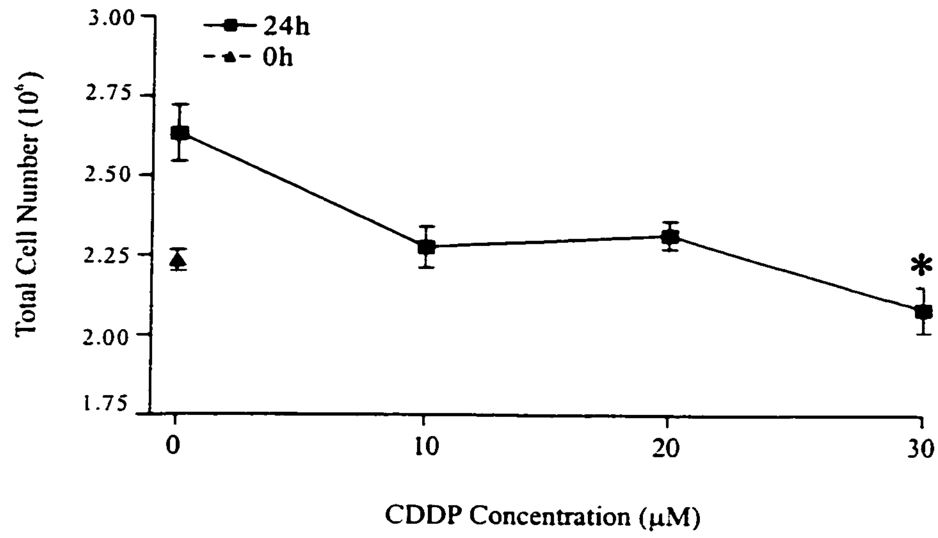


Figure 7. Effect of CDDP and agonistic Fas Ab on cell number in OV2008 hOSE cancer cells.

Concentration-dependent effect of CDDP (A) and agonistic Fas Ab (B) on cell number in OV2008 cells. CDDP (0-30 μM; A) or agonistic Fas Ab (2 μg/ml; B) was added to OV2008 cultures for 24h. Cells were then trypsinized and counted using a hemocytometer. N=3 experiments; * $p<0.05$ (compared to control).

A



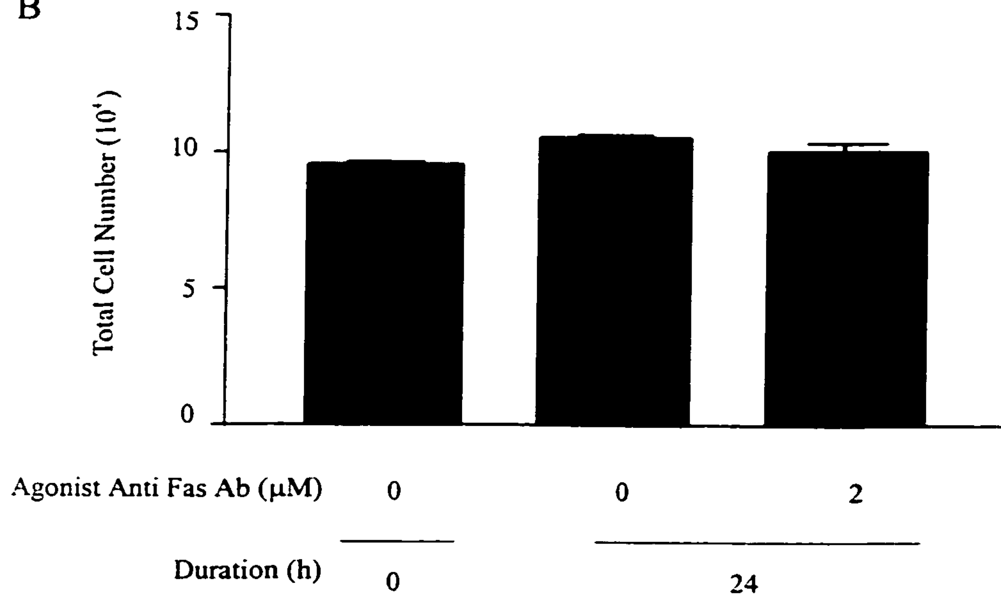
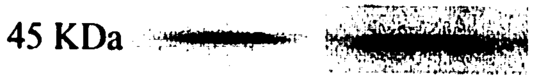
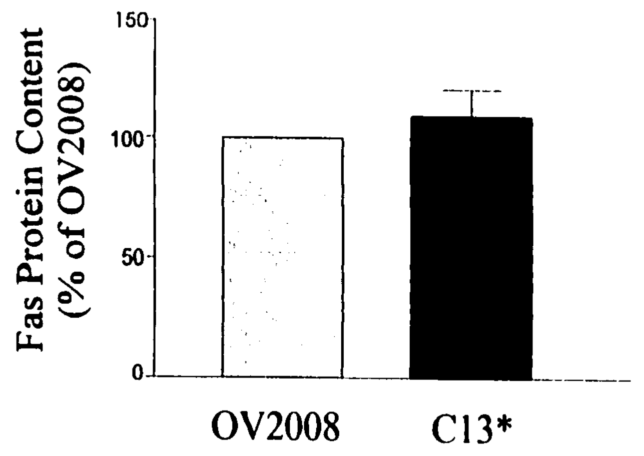


Figure 8. Basal Levels of Fas and FasL in OV2008 (sensitive) and C13*(resistant) hOSE Cancer Cells.

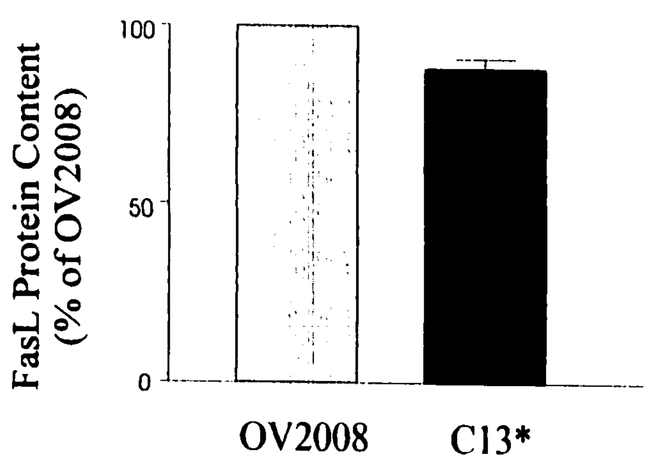
After plating, hOSE cancer cells were cultured in the presence of 10% FBS for 24h. Total cell lysates were sonciated, resolved on SDS-PAGE, electrotransferred onto nitrocellulose membrane and subjected to ECL. 30 μg of total protein was loaded onto each lane. Monoclonal human anti-Fas and anti-FasL Abs were used. Data expressed as mean % of OV2008 cells ± SEM; N=3 experiments.

Fas





FasL



14.3 CDDP Increased Fas and FasL Protein Contents

Fas and FasL protein expression was detectable by Western analysis in both OV2008 and C13*. Basal levels of these proteins were not significantly different between the CDDP-resistant and -sensitive cells (Fig 8). To establish the role and regulation of Fas and FasL in CDDP-induced apoptosis and their possible involvement in chemoresistance, OV2008 and C13* were cultured in the absence and presence of CDDP (10-30μM) and its influence on Fas and FasL expression and apoptosis was assessed. In the absence of cisplatin, Fas and FasL were present in both cell types as 45 and 37 kDa proteins, respectively. CDDP significantly increased cell-associated Fas protein content in both OV2008 and C13* in a concentration-dependent manner (Fig. 9). Whereas cell-associated FasL expression in the sensitive cells was also increased after CDDP treatment, there was no significant change in the protein content of the ligand in the resistant ones even at concentrations up to 30μM (Fig. 9). The increases in FasL and Fas protein content, in OV2008, were time-dependent with significant effects of CDDP (20μM and 30μM) observable at 12 and 24h, respectively ($p<0.05$; Fig. 10 A and 10 A). In contrast, the increase in Fas protein content in C13* cells in the presence of a high CDDP concentration (30μM) was transient, while Fas content in the presence of a lower concentration of the anti-cancer agent (20μM) were related to the duration of the culture period ($p<0.01$; 10 B). The levels of cell-associated FasL were not significantly affected by the presence of CDDP, irrespective of its concentration or the duration of culture period ($p>0.05$; Fig. 11 B). Jurkat cells were used as a positive control for human Fas protein and human endothelial cells were used as a positive control for human FasL protein.

14.4 CDDP increased sFasL levels in OV2008 but not C13* hOSE cancer cell cultures

Figure 9. Concentration dependent effect of CDDP on Fas and FasL protein contents.

Western analyses illustrating cell-associated Fas and FasL levels in CDDP-sensitive (OV2008) and -resistant (C13*) cells cultured for 24h in the presence of different concentrations of CDDP. Panel A illustrates representative filters and Panel B indicates changes in protein content as analyzed densitometrically. Data is expressed as mean % of control ± SEM; N=3 experiments. *$p<0.05$ (compared to control).

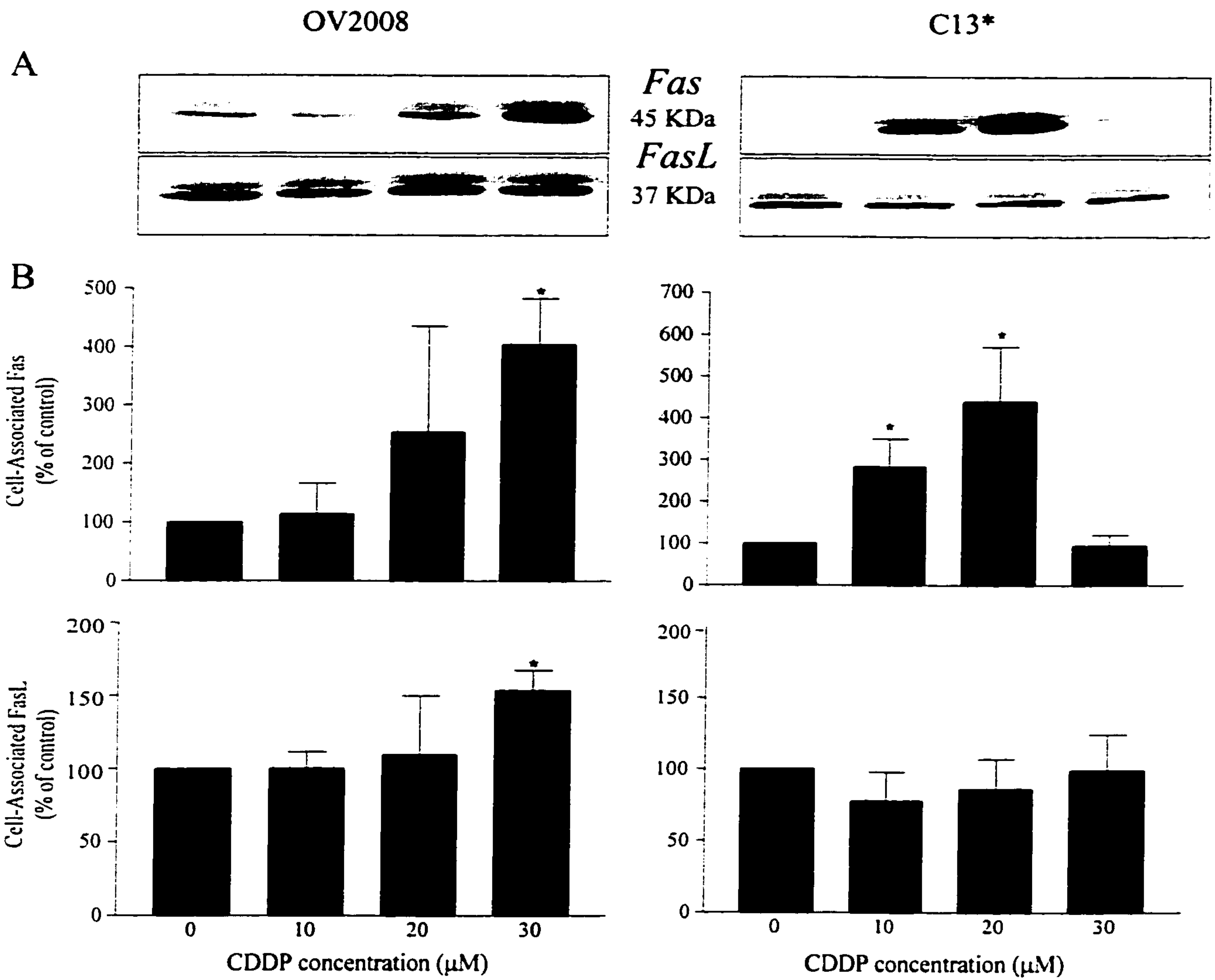
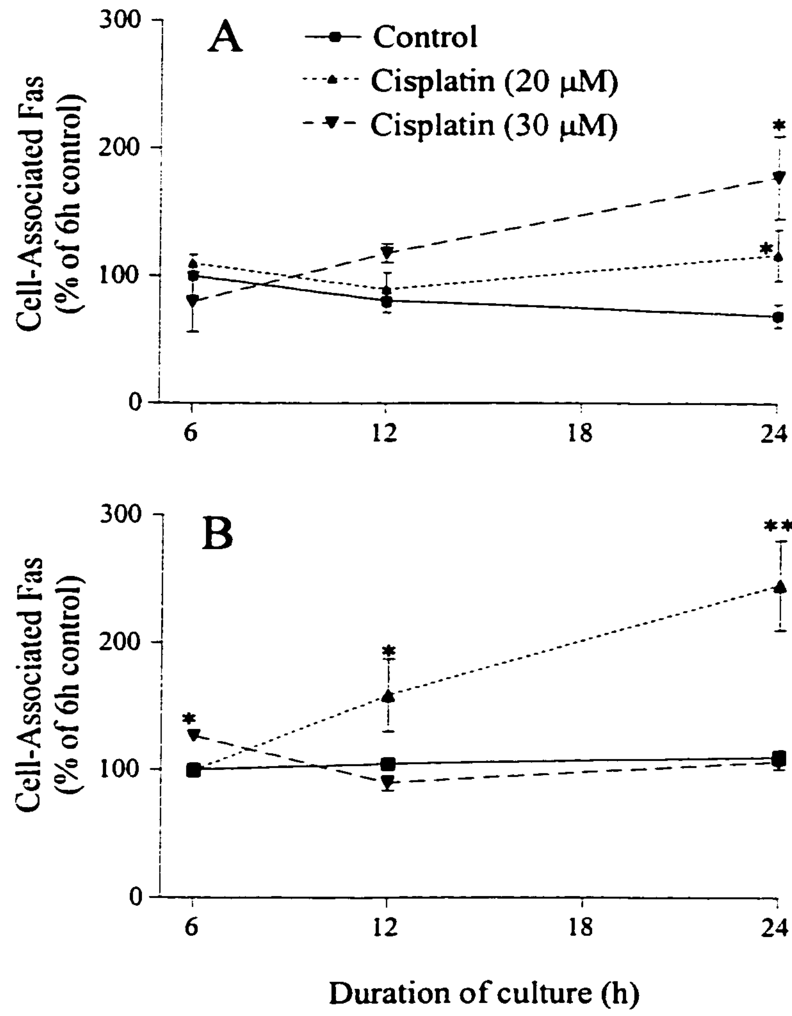

OV2008
C13*
A
Fas
45 KDa
FasL
37 KDa
B
Cell-Associated Fas
(% of control)
Cell-Associated FasL
(% of control)
0
10
20
30
CDDP concentration (μM)
CDDP concentration (μM)

Figure 10. Time-dependent effect of CDDP on Fas protein levels in OV2008 and C13* hOSE cancer cells.

Temporal changes in cell-associated Fas content in OV2008 (A) and C13* (B) cells cultured in the absence and presence of CDDP (20 and 30 μM). Bottom panels indicate changes in protein content as analyzed densitometrically. Data is expressed as mean % of 6h control ± SEM; N=3 experiments. *$p<0.05$, **$p<0.01$ (compared to 6h-control)

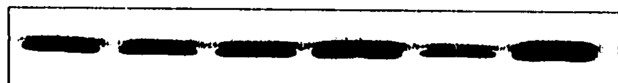


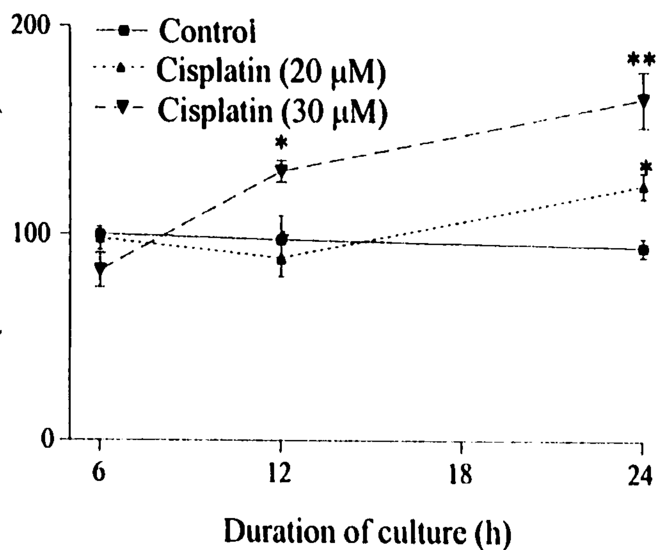
A
Control
Cisplatin (20 μM)
Cisplatin (30 μM)
Cell-Associated Fas
(% of 6h control)
300
200
100
0
6
12
18
24
*
*
B
**
*
*
Duration of culture (h)

Figure 11. Time-dependent effect of CDDP on FasL protein levels in OV2008 and C13* hOSE cancer cells

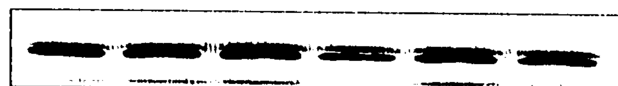
Temporal changes in cell-associated FasL contents in OV2008 (A) and C13* (B) cells cultured in the absence and presence of CDDP (20 and 30μM). Bottom panels indicate changes in protein content as analyzed densitometrically. Data is expressed as mean % of 6h control ± SEM; N=3 experiments. *$p<0.05$, **$p<0.01$ (compared to 6h-control)

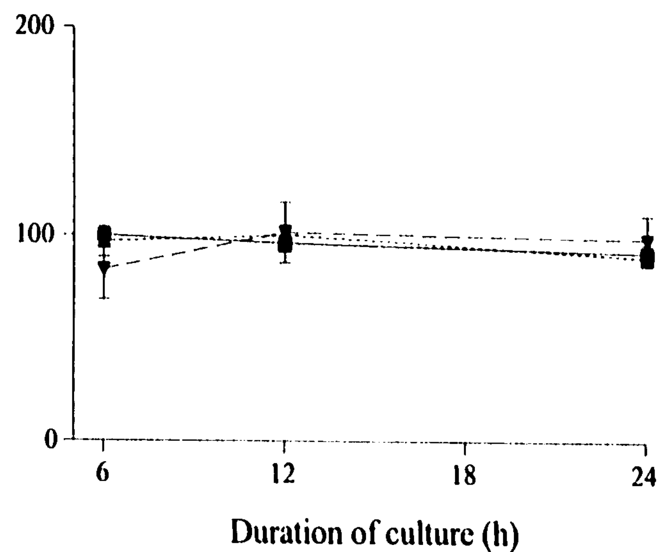
A





B



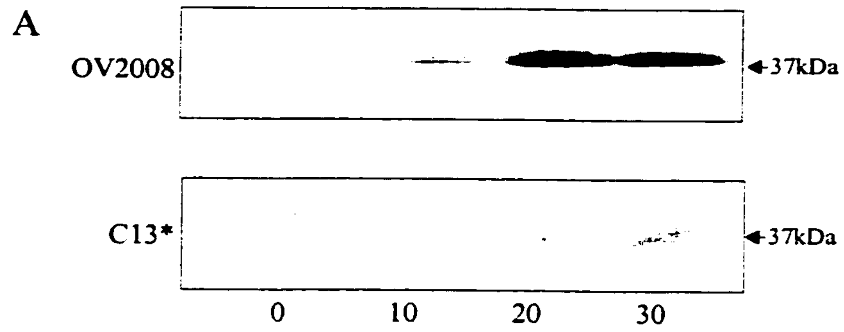


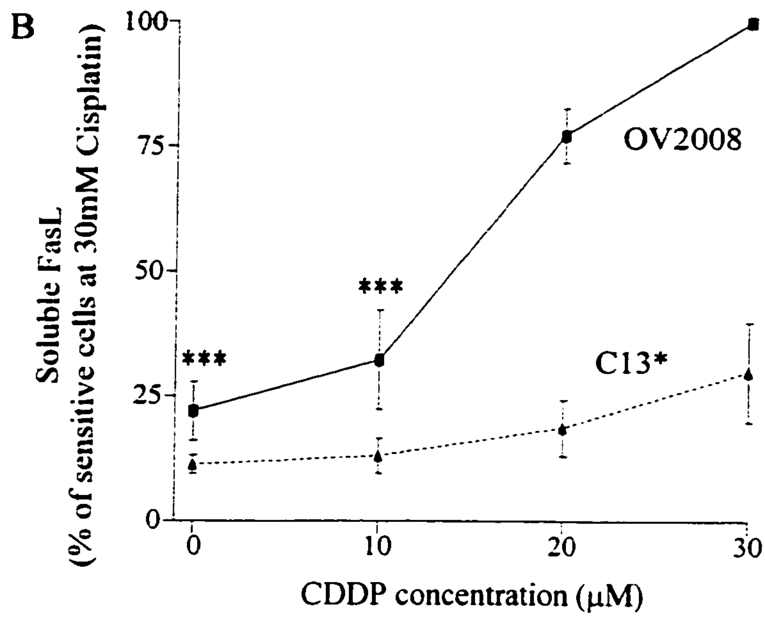
It has been demonstrated that certain disease states are associated with altered levels of soluble FasL in tissue and serum (Tanaka et al 1996). If FasL is solublized, it can act in a autocrine and paracrine manner to induce cell death. To determine if soluble FasL is indeed present and its expression regulated in hOSE cell cultures by CDDP and related to CDDP sensitivity, the effects of CDDP on the levels of FasL in the media of OV2008 and C13 cultures were compared (Fig. 12). As observed with the changes in cell-associated FasL content in both sensitive and resistant cells, CDDP significantly induced a concentration-dependent increase in the levels of soluble FasL (37kDa) in OV2008 ($p<0.001$; Fig. 12 A and B) but not C13* cells ($p>0.05$; Fig. 12 A and B). At 30 μM CDDP, the levels of soluble FasL in the resistant variant were only about 20% of those in the sensitive cells ($p<0.001$; Fig. 12 B). To clarify whether the FasL present in the spent media could have been associated with cellular debris release during cell culture or harvest, FasL levels in spent media which underwent filtration only and filtration following ultracentrifugation were compared. There was no significant difference between the two methods ($p>0.05$; Fig. 13), suggesting that filtration alone was sufficient in removing possible debris from the medium. As shown in Fig 12C, the levels of Fas in the media were low yet similar between OV2008 and C13* hOSE cancer cells, and remained unchanged with CDDP treatment. These results suggest that chemoresistance may be associated with an inability of the cells to express and release significant amounts of FasL in response to CDDP.

To assess whether the FasL detected in the spent media from OV2008 cultures treated with CDDP was biologically active and able to induce cell death, concentrated spent media containing FasL were added to C13* cell cultures. Although most of the CDDP in the media would have been filtered out during the concentration process, C13* cells (instead of OV2008 cells) were used as a test cell since trace amounts of CDDP in the concentrated media alone would not have caused apoptosis in the C13* cells. In addition, C13* and OV2008 contain similar amounts of Fas (Figure 8) and are equally

Figure 12. Concentration-dependent effects of CDDP on soluble FasL and Fas protein levels in hOSE cancer cells cultures.

Western blot showing CDDPmarkedly increased immunoreactive FasL levels in spent media of CDDP-sensitive (OV2008) but not -resistant (C13*) cells cultured for 24h. Panel A illustrates representative filters and Panel B indicates changes in protein content as analyzed densitometrically. Panel C illustrates representative filters of Fas receptor detected in the media after CDDP treatment. Data are expressed as a percentage of sensitive cells cultured in the presence of 30 μM CDDP and represent mean ± SEM of three experiments. *$p<0.05$; **$p<0.01$ (compared to OV2008 at 30μM cisplatin).


A
OV2008
37kDa
C13*
37kDa
0
10
20
30


B
100
75
50
25
0
Soluble FasL
(% of sensitive cells at 30mM Cisplatin)
OV2008
C13*
***
***
0
10
20
30
CDDP concentration (μM)

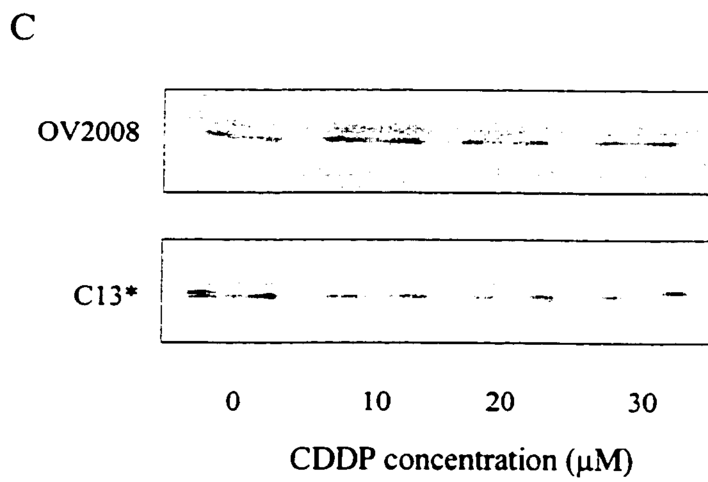
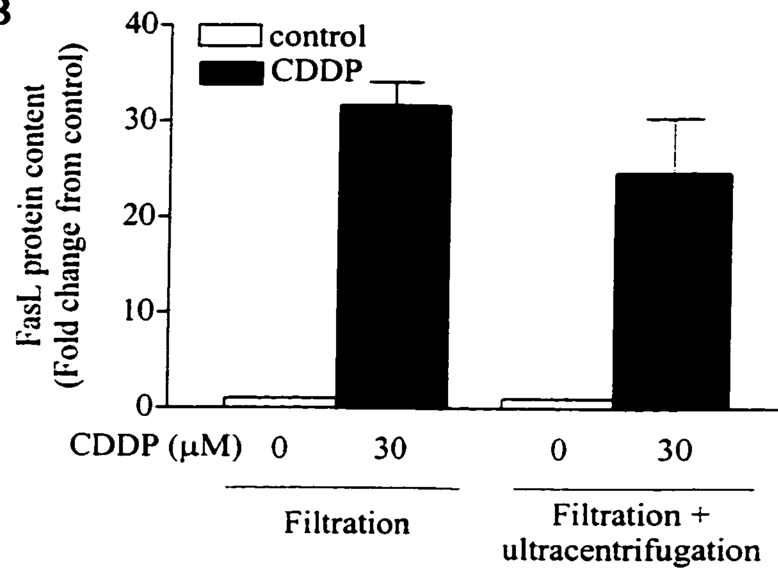

C
OV2008
C13*
0
10
20
30
CDDP concentration (μM)

Figure 13. Filtration vs Filtration and Ultracentrifugation on FasL in spent media OV2008 cultures

Spent media from CDDP (30μM) treated OV2008 cell cultures was subject to filtration alone or filtration followed by ultracentrifugation (393, 201 x g; 30 min). The media was concentrated (48x) using a centricon spin column and the protein was subject to Western analysis. Panel A illustrates representative filters and Panel B indicates changes in protein content as analyzed densitometrically. Data are expresssed as fold change from control ± SEM; N=3 experiments.

A

B



responsive to agonist Fas Ab (Fig.16). Concentrated (48X) media from OV2008 was able to significantly increase cell death in C13* cells from 2.1% ± 0.46 to 9.4 % ± 1.2 ($p<0.001$; Fig. 14). Addition of the antagonistic Ab significantly reduced the number of cells undergoing spent medium-induced apoptosis by 56% ($p<0.05$; Fig. 14). The presence of FasL in the concentrated media did not significantly increase apoptosis induced by the addition of agonistic Fas mAb (Fig. 14). These findings indicate that one of the death factors in the spent media was indeed FasL and that it in part contributes to CDDP-induced apoptosis in the ovarian cancer cells.

14.5 The effects of CDDP on Fas, FasL, sFas in A2780-s and A2780-cp hOSE cancer cells

To ascertain if the defective regulation of the Fas/FasL system in C13* cells in response to CDDP is not unique to this ovarian cancer cell line, another CDDP-sensitive (A2780-s) and -resistant (A2780-cp) hOSE cancer cell lines were examined. CDDP (30μM) significantly increased cell-associated Fas and FasL ($p<0.05$; Fig. 15a) and soluble FasL ($p<0.01$; Fig. 15c) contents in A2780-s but not in its resistant variant (A2780-cp; $p>0.05$; Fig. 15 a,b and c) during a 24h culture period. Lower concentrations of CDDP were effective in inducing Fas expression in A2780-s, with 20 μM CDDP Fas content was 1.52 ± 0.10 fold over control ($p<0.05$; n=3). However, concentrations of CDDP below 30 μM were not effective in inducing Fas expression in A2780-cp cells, with 3 , 10 and 20 μM CDDP, Fas content was 1.02 ± 0.17, 1.0 ± 0.10 and 0.89 ± 0.08 fold of control, respectively ($p>0.05$; n=3). Although apoptosis in the resistant cells was

Figure 14. Assessment of sFasL activity on C13* hOSE cancer cells

Media from OV2008 cells treated with CDDP (30 μM) for 24h was filtered and concentrated (48X) using a centricon spin column and added to subconfluent cultures of C13* cells in serum-free media for 24h either alone, in the presence of agonistic Fas mAb (2μg/ml) or after pretreatment (6h) with an antagonistic Fas mAb (10μg/ml). N=3 experiments; ** $p<0.001$ (compared to control) and * $p<0.05$ (compared to spent media)

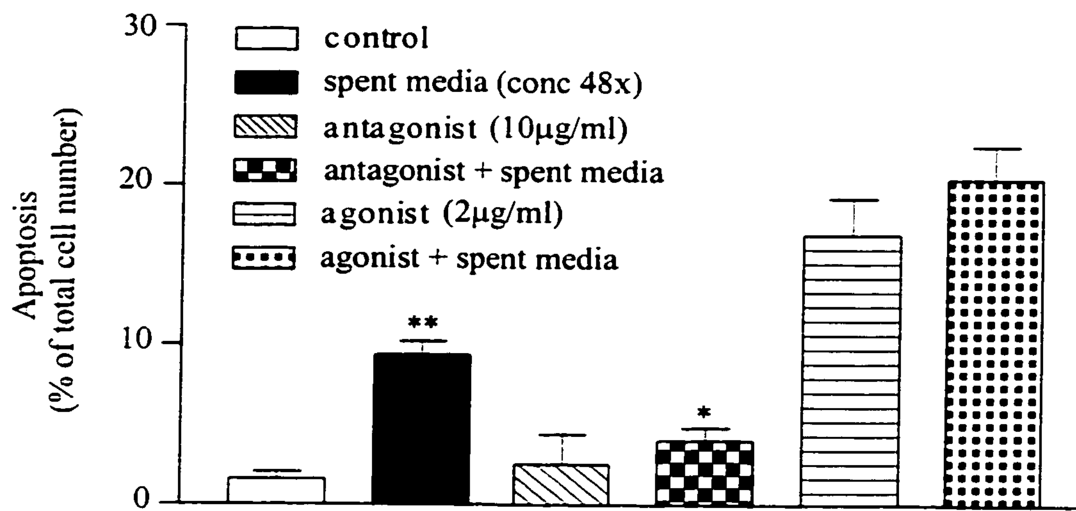
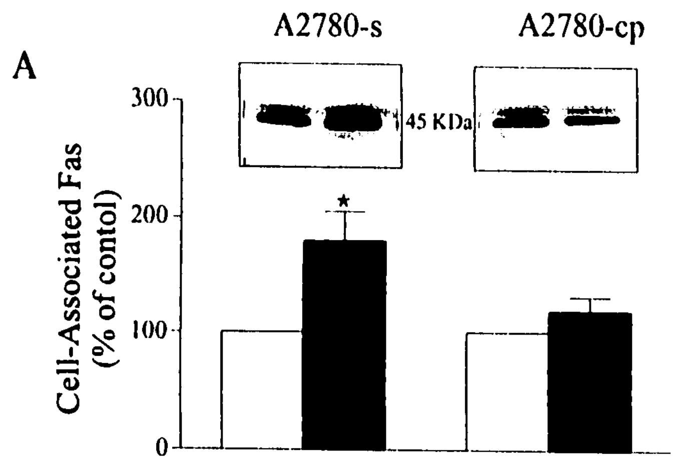
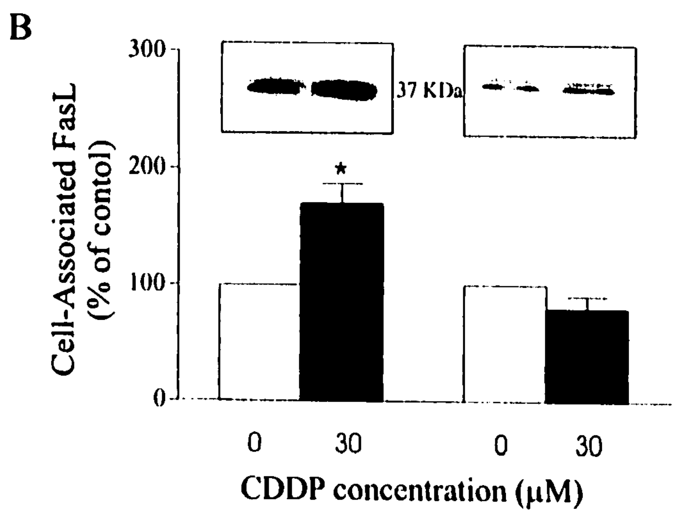
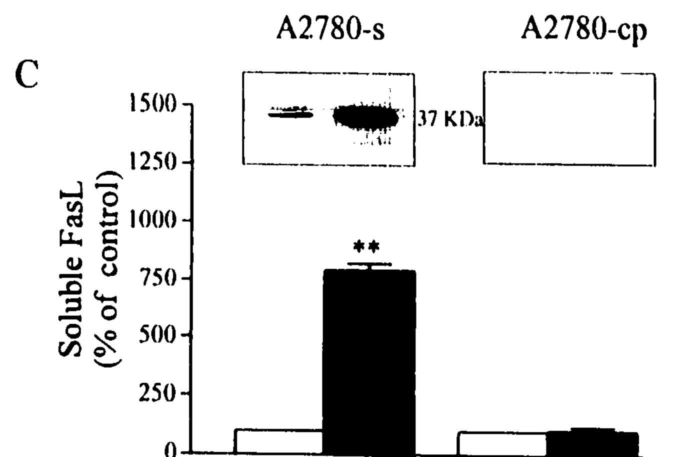

30
20
10
0
Apoptosis
(% of total cell number)
control
spent media (conc 48x)
antagonist (10μg/ml)
antagonist + spent media
agonist (2μg/ml)
agonist + spent media
**
*

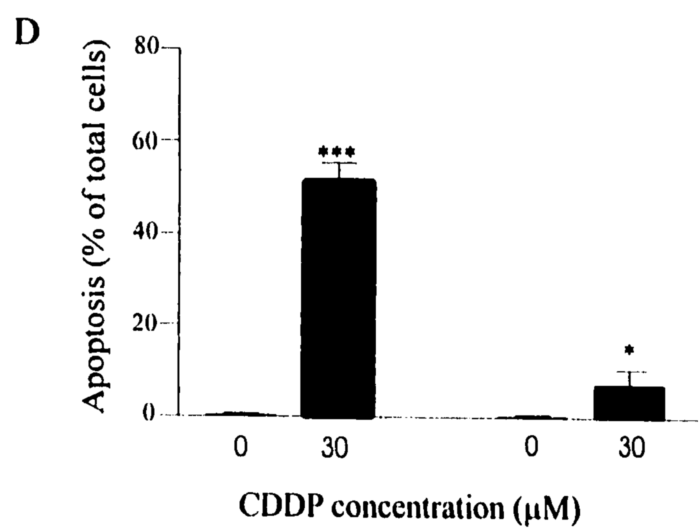
Figure 15. Effects of CDDP on Fas, FasL and sFasL in A2780-s and A2780-cp hOSE cancer cells.

Western blot illustrating differential effects of CDDP (30μM) on cell-associated Fas (Panel A) and FasL (Panel B), soluble FasL (Panel C) protein contents and apoptosis (Panel D) in a CDDP-sensitive (A2780-s) and -resistant (A2780-cp) cell line for 24h. Each panel shows a representative filter (top) and mean changes in protein content as analyzed densitometrically (bottom). Data represent mean ± SEM of three experiments. *$p<0.05$, **$p<0.01$ (compared to respective control).


A
A2780-s
A2780-cp
45 KDa
Cell-Associated Fas (% of contol)
300
200
100
0
*


B
37 KDa
Cell-Associated FasL (% of contol)
300
200
100
0
*
0
30
0
30
CDDP concentration (μM)


C
A2780-s
A2780-cp
37 KDa
Soluble FasL (% of control)
1500
1250
1000
750
500
250
0
**


D
Apoptosis (% of total cells)
80
60
40
20
0
***
*
0
30
0
30
CDDP concentration (μM)

significantly increased ($p<005$) in the presence of CDDP, the extent was minimal compared to that observed in the sensitive cells (Fig 15d).

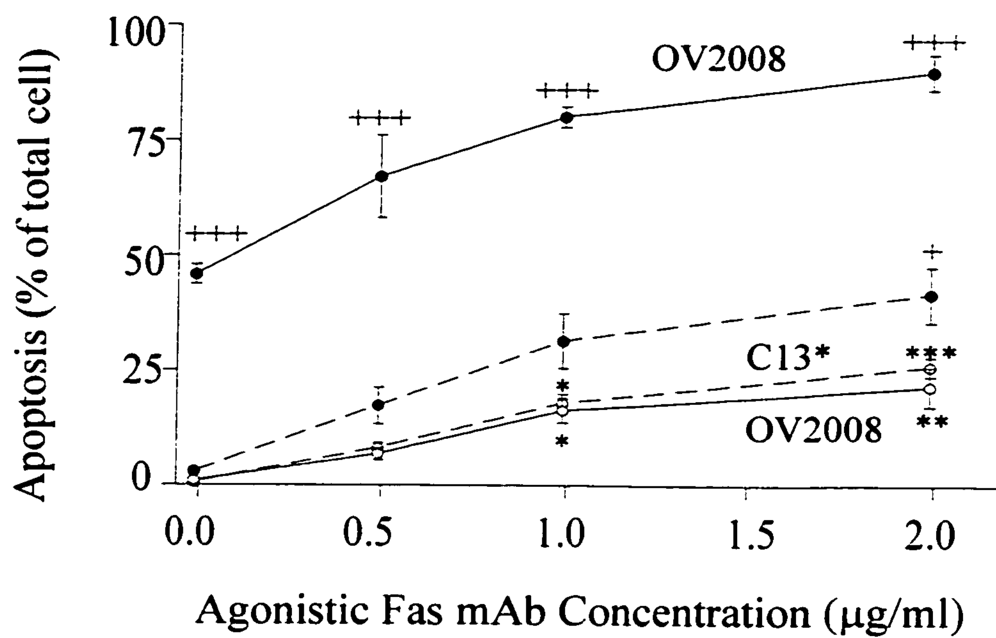
14.6 CDDP and agonistic Fas Ab exhibit a synergistic effect on apoptosis

To further investigate the role of Fas in CDDP-induced apoptosis in ovarian cancer cells and whether inadequate expression and release of sFasL in the resistant cells (C13*) following CDDP treatment could be contributing to their overall chemo-resistance, OV2008 and C13* cells were challenged with different concentrations of an agonistic Fas Ab (0-2.0 μg/ml; to mimick FasL). There was a concentration-dependent increase in apoptosis in both OV2008 and C13* after activation of the Fas receptor ($p<0.01$; Fig. 16). The level of cell death induced by the agonistic Fas Ab was not significantly different between the two cells lines ($p>005$). This is consistent with our earlier data indicating that both cell types contain similar levels of the Fas receptor protein (Fig.8). Since both cell types are equally responsive to agonist Fas Ab challenge, the postreceptor signaling events are intact in both CDDP-sensitive and -resistant cells. Analysis of variance indicates that there was a significant interaction between CDDP and Fas Ab effects in the sensitive cells, brought about by a greater cell kill effect with this combination treatment ($p<0.05$; Fig16). There was a higher incidence of apoptosis in the C13* cell line with the combination of CDDP and agonist Ab ($p<0.001$) than either one alone. The observed increase in cell death with the combination of CDDP and Fas mAb was significantly higher in OV2008 than in C13* ($p<0.01$; Fig. 16).

14.7 CDDP-induced apoptosis is accompanied by caspase-8 cleavage

Figure 16. Effects of CDDP and Agonistic Fas Ab on apoptosis in OV2008 and C13* hOSE cancer cells.

Influence of Fas mAb (0-2μg/ml) and CDDP (10μM), alone or in combination, on the incidence of apoptosis in OV2008 and C13* during a 24h culture. (__), OV2008 cells; (---), C13* cells; (○), in the absence of CDDP; (●), in the presence of CDDP. Data represent mean ± SEM of three experiments. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ (compared to respective control); + $p<0.05$ +++ $p<0.001$ (compared to cells cultured in the absence of CDDP and at same Fas mAb concentration)


100
75
50
25
0
Apoptosis (% of total cell)
+++
OV2008
+++
+++
+++
+
C13*
***
*
*
OV2008
**
0.0
0.5
1.0
1.5
2.0
Agonistic Fas mAb Concentration (μg/ml)

If CDDP induced apoptosis involves the interaction of Fas/FasL, cleavage of caspase-8 should be detectable after CDDP challenge. CDDP treatment results in the cleavage of caspase-8 into its active p18 fragment in OV2008 cells, with maximal cleavage observable at 10μM (Fig. 17). In the presence of CDDP (30μM), the prodomain of caspase-8 decreased significantly in the 24h cultures of OV2008 ($p<0.01$; Fig. 17 and 18). The appearance of a p43 cleavage product appeared after 12h of culture ($p<0.01$) and was maximal at 24h. A p18 product was also observed in OV2008 at 24h (Fig. 18). There was no detectable decrease in the pro-caspase-8 protein and appearance of cleavage products with duration of culture of C13* cells (Fig. 17 and 18).

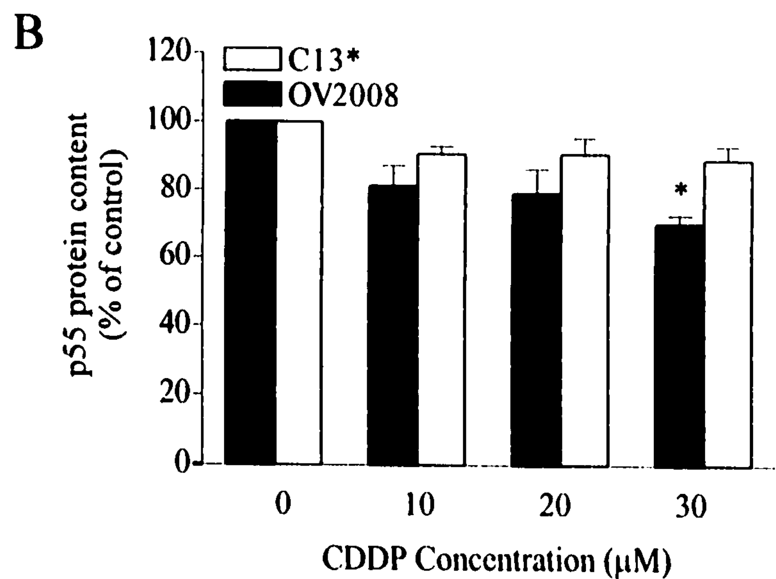
14.8 CDDP-induced apoptosis is accompanied by caspase-3 cleavage in OV2008 hOSE cancer cells.

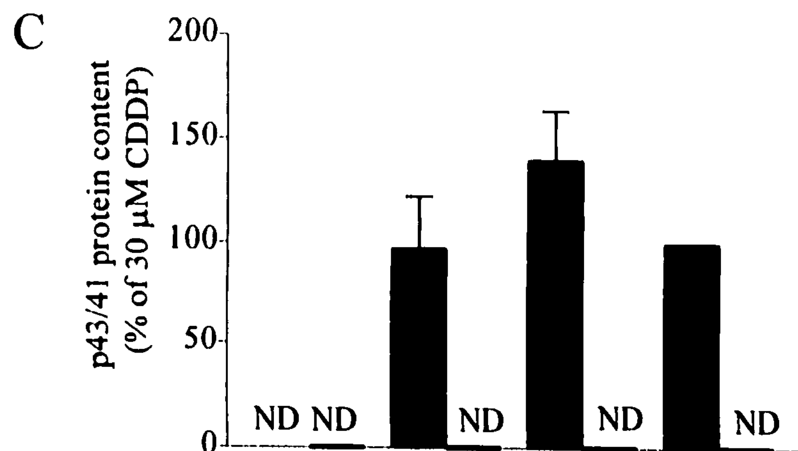
Fas activation results in caspase-8 cleavage and activation which then leads to caspase-3 cleavage and activation (Scaffidi et al 1998). It has recently been demonstrated that caspase-3 can be activated in response to DNA-damage (Fuch et al 1997). In order to determine if caspase-3 is activated after CDDP treatment in OV2008 and C13*, Western analyses were performed to assess the influence of CDDP on the cellular contents of caspase-3 (p32) and its active cleavage products p20 and p17. CDDP decreased caspase-3 protein content (p32) in OV2008 ($p<0.05$; Fig 20 and 21) but not in C13* (Fig 20 and 22) in a concentration- and time-dependent manner. Coincidently, the levels of the active cleavage products of caspase-3, p17 and p20 were also increased ($p<0.0001$ for both fragments; Fig. 20 and 21). There were no detectable caspase-3 cleavage fragments in C13* cells after CDDP treatment irrespective of the duration of culture (Fig. 20 and 22) although the presence of these active fragments was evident in OV2008 cells at 12h of culture and to a greater extent at 24h ($p<0.05$; Fig. 21).

Figure 17. Effect of different concentrations of CDDP on the cleavage of caspase-8 in OV2008 and C13* hOSE cancer cells.

Cells were cultured with CDDP (0-30 μM) for 24 h. Total proteins were extracted and resolved by SDS-PAGE. ECL was performed using monclonal human anti-caspase-8 Ab that recognizes caspase-8 and its cleavage products (except p10). Panel A, representative filters and Panel B corresponding densitometric analysis of protein fragments. Data are expressed as mean % of control (Panel B) and as % of 30 μM CDDP (Panels Cand D) ± SEM; N=3 experiments. * $p<0.05$.

A
OV2008
C13*
0
10
20
30
CDDP (μM)
◄ 55 kDa ►
◄41/43 kDa►
◄ 18 kDa ►


B
p55 protein content
(% of control)
120
100
80
60
40
20
0
C13*
OV2008
*
0
10
20
30
CDDP Concentration (μM)


C
p43/41 protein content
(% of 30 μM CDDP)
200
150
100
50
0
ND
ND
ND
ND
ND

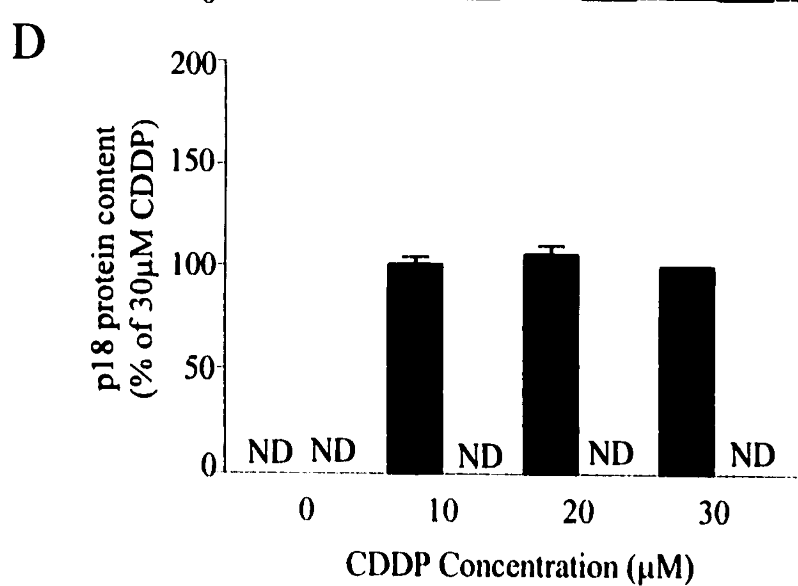
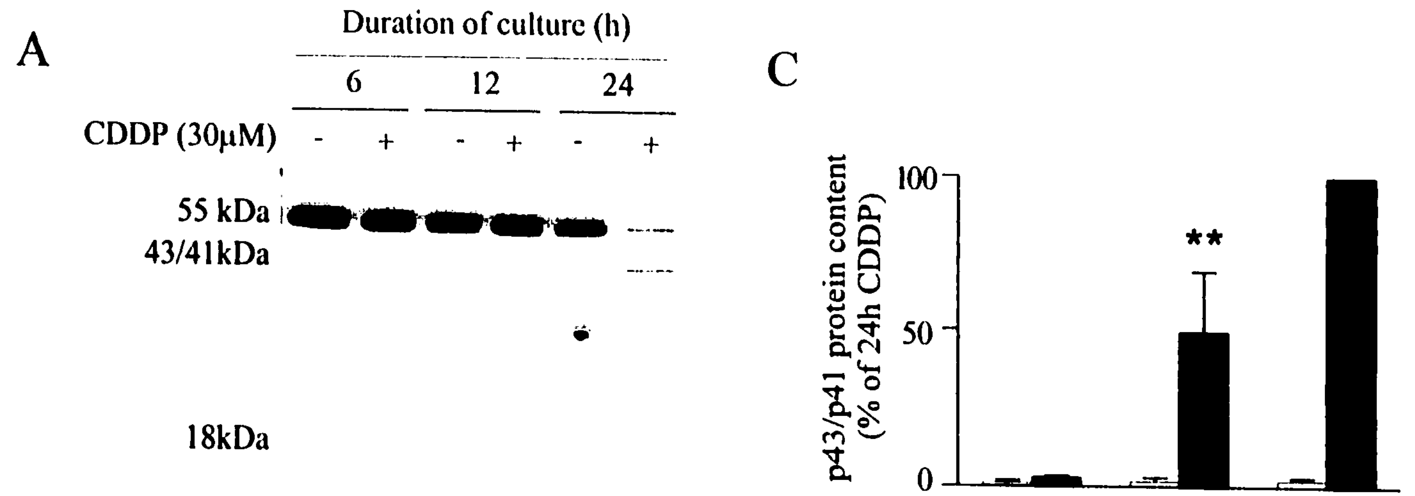
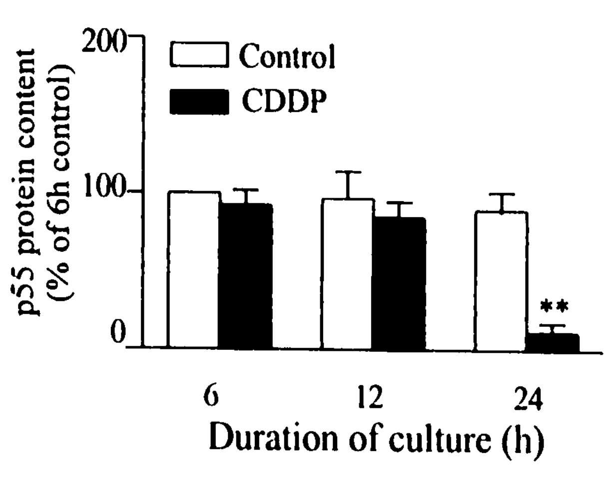

D
p18 protein content
(% of 30μM CDDP)
200
150
100
50
0
ND
ND
ND
ND
ND
0
10
20
30
CDDP Concentration (μM)

Figure 18. Time Course of cleavage of caspase-8 in OV2008 hOSE cancer cells induced by CDDP.

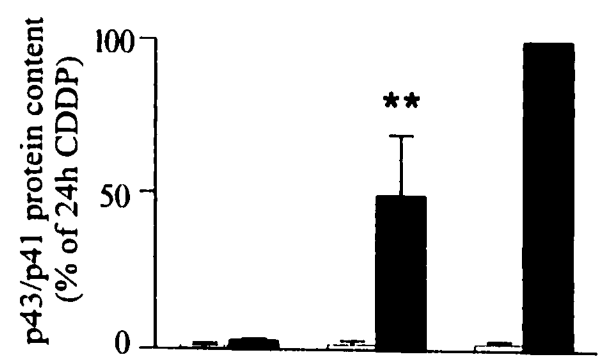
CDDP (30μM) was added to OV2008 cultures for up to 24h. Total cell protein was resolved on SDS-PAGE and electrotransferred onto nitrocellulose. ECL was performed using a monclonal human anti-caspase-8 Ab which recognizes caspase-8 (p55) and its cleavage products (p43/41 and p18). Panel A, representative filter; Panel B, densitometric analysis. Data are expressed as a mean % of 6h control ± SEM; N=3 experiments. ** $p<0.01$. (compared to respective control)



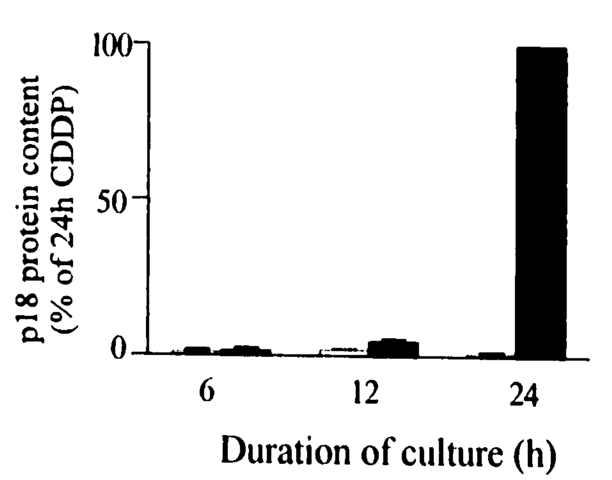
A
Duration of culture (h)
6
12
24
CDDP (30μM)
- + - + - +
55 kDa
43/41kDa
18kDa



B
p55 protein content
(% of 6h control)
200
100
0
Control
CDDP
**
6
12
24
Duration of culture (h)



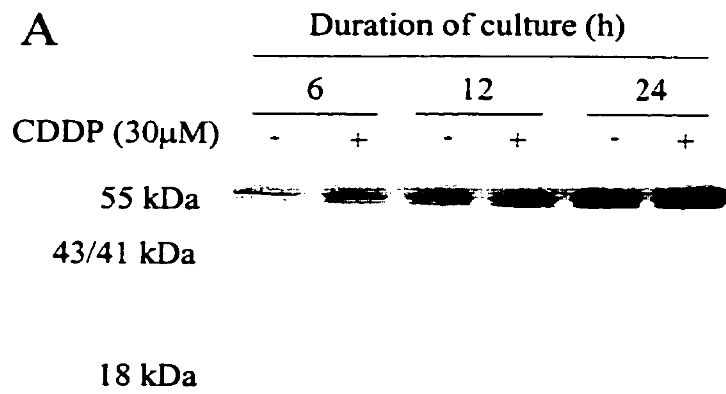
C
p43/p41 protein content
(% of 24h CDDP)
100
50
0
**



D
p18 protein content
(% of 24h CDDP)
100
50
0
6
12
24
Duration of culture (h)

Figure 19. Lack of effect of CDDP on caspase-8 cleavage in C13* hOSE cancer cells.

CDDP (30μM) was added to C13* cultures for up to 24h. Total cell protein was resolved on SDS-PAGE and electrotransferred onto nitrocellulose. ECL was performed using a monclonal human anti-caspase-8 Ab which recognizes caspase-8 (p55) and its cleavage products (p43/41 and p18). Panel A, representative filter; Panel B, densitometric analysis. Data are expressed as a mean % of 6h control ± SEM; N=3 experiments.


A
Duration of culture (h)
6
12
24
CDDP (30μM)
-
+
-
+
-
+
55 kDa
43/41 kDa
18 kDa

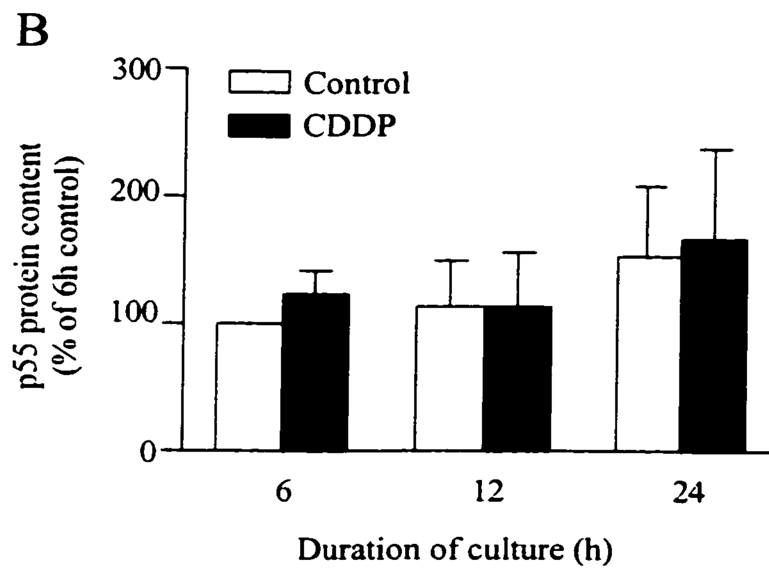
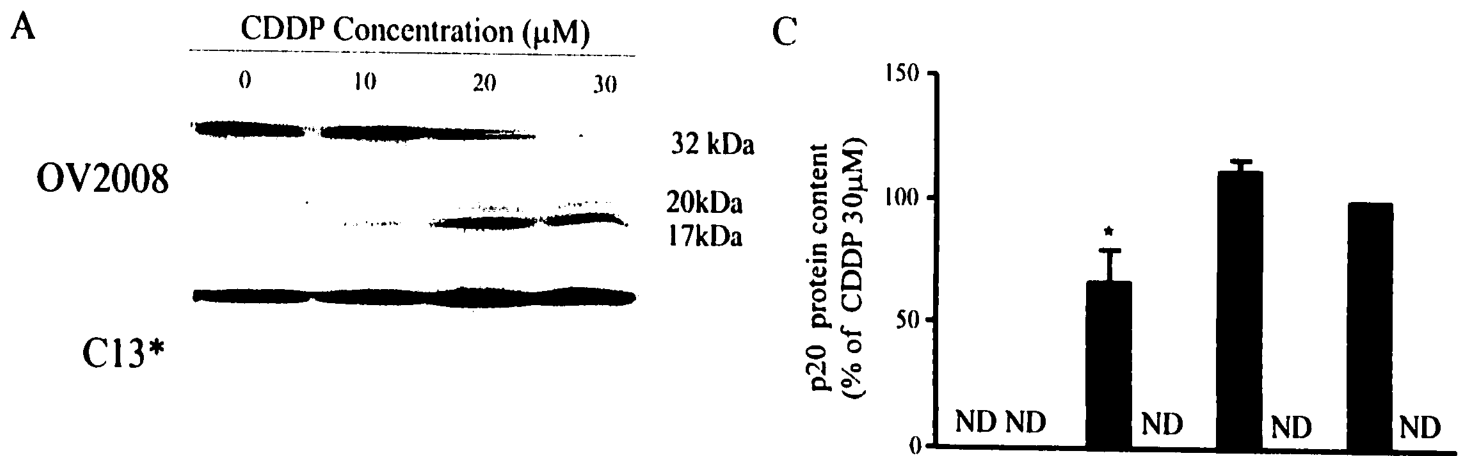

B
300
200
100
0
Control
CDDP
p55 protein content
(% of 6h control)
6
12
24
Duration of culture (h)

Figure 20. Concentration-dependent effects of CDDP on caspase-3 cleavage in OV2008 and C13* hOSE cancer cells.

CDDP (10-20 μM) was added to hOSE cancer cultures for 24h. Total cell protein was resolved on SDS-PAGE and electrotransferred onto nitrocellulose. ECL was performed using a polyclonal human anti-caspase-3 Ab that recognizes caspase-3 (p32) and its cleavage products (p20 and p18). Panel A, representative filter; Panel B, densitometric analysis. Data are expressed as a mean % of control (Panel B) or 30 μM CDDP group (Panels C and D) ± SEM; N=3 experiments. * $p<0.05$.


A
CDDP Concentration (μM)
0
10
20
30
OV2008
C13*
32 kDa
20kDa
17kDa
B
Prodomain p32 protein content
(% of control)
150
100
50
0
OV2008
C13*
*
*
CDDP Concentration (μM)

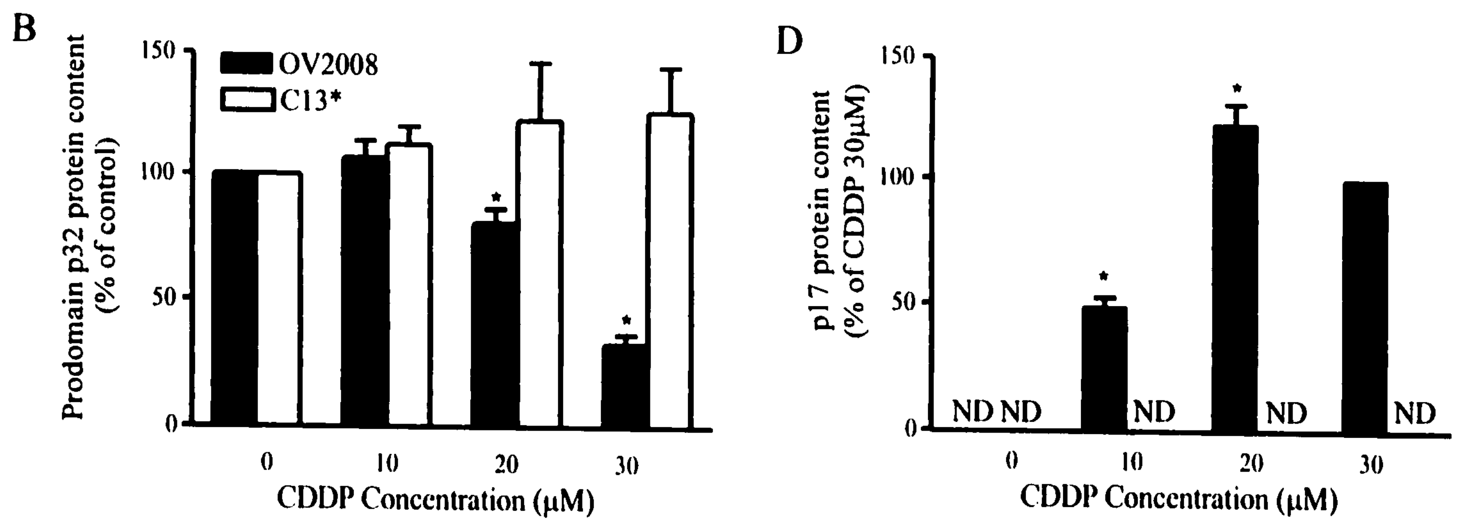
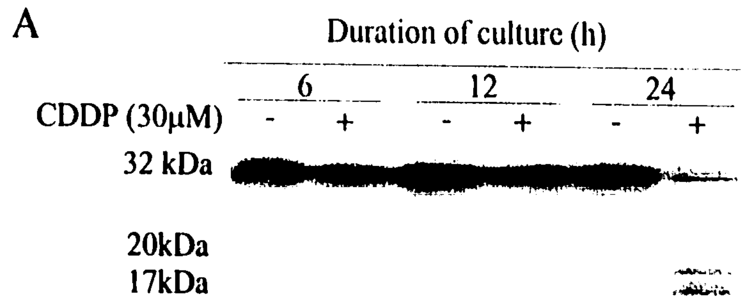
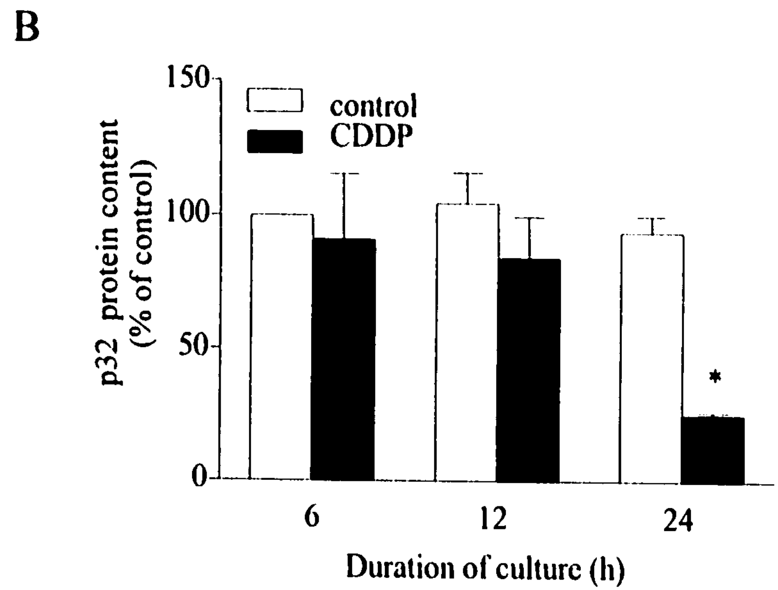
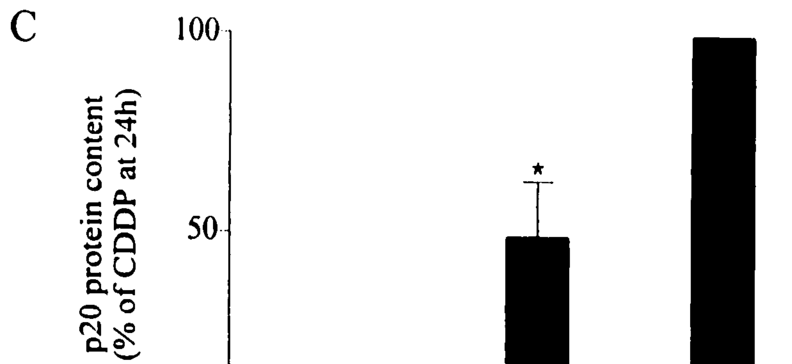

C
p20 protein content
(% of CDDP 30μM)
150
100
50
0
ND ND
ND
ND
ND
*
D
p17 protein content
(% of CDDP 30μM)
*
*
0
10
20
30
CDDP Concentration (μM)

Figure 21. Temporal effects of CDDP on caspase-3 cleavage in OV2008 hOSE Cancer cells.

CDDP (30μM) was added to OV2008 cultures for up to 24h. Total cell protein was resolved on SDS-PAGE and electrotransferred onto nitrocellulose. ECL was performed using a polycolonal human anti-caspase-3 Ab that recognizes caspase-3 (p32) and its cleavage products (p20/17). Panel A, representative filter Panels B-D, densitometric analysis. Data are expressed as a mean % of 6h control (Panel B) or of CDDP group at 24 h (Panels C and D) ± SEM; N=3 experiments. * $p<0.05$.


A
Duration of culture (h)
6
12
24
CDDP (30µM)
-
+
-
+
-
+
32 kDa
20kDa
17kDa


B
control
CDDP
p32 protein content
(% of control)
150
100
50
0
*
6
12
24
Duration of culture (h)


C
p20 protein content
(% of CDDP at 24h)
100
50
0
*

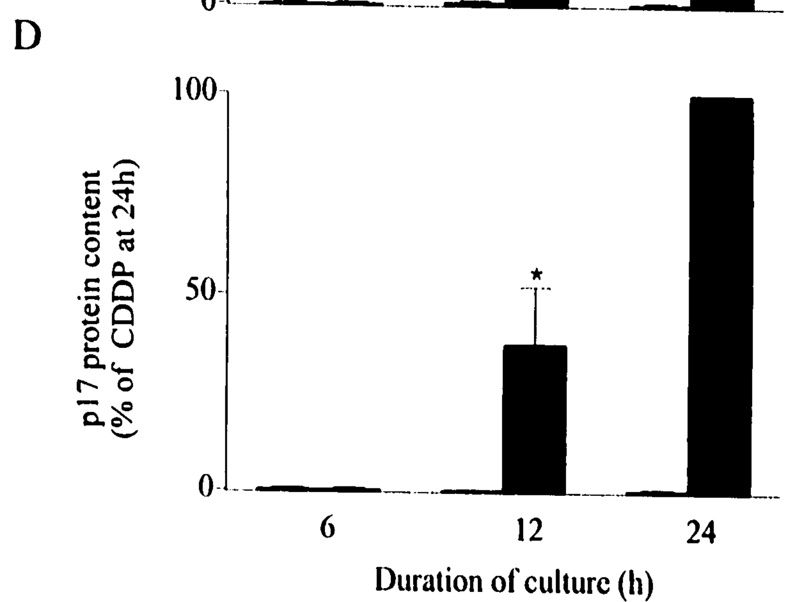
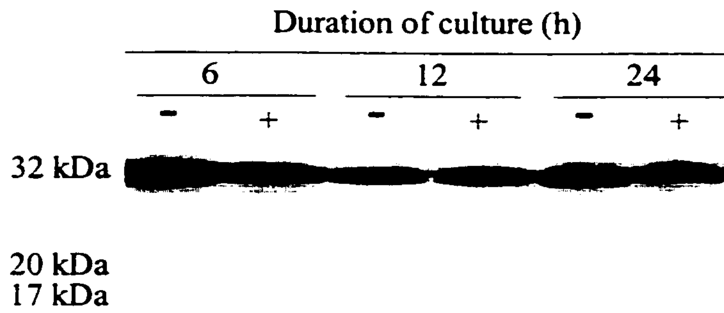
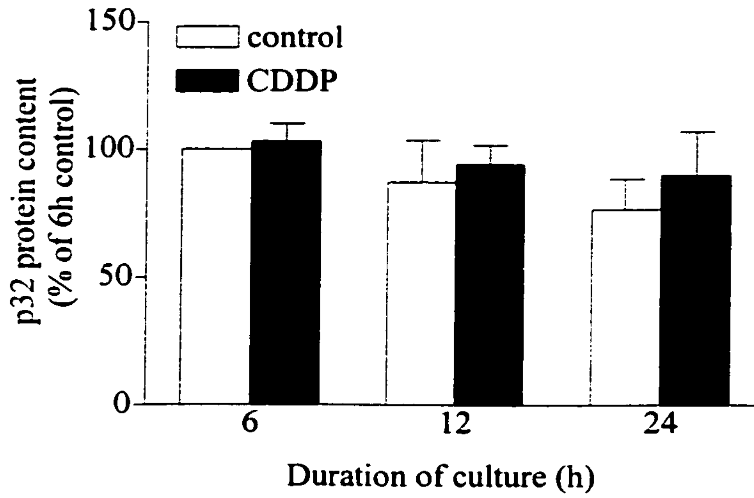

D
p17 protein content
(% of CDDP at 24h)
100
50
0
*
6
12
24
Duration of culture (h)

Figure 22. Lack of effect of CDDP on caspase-3 cleavage in the CDDP-resistant cell line (C13*).

CDDP (30μM) was added to C13* cultures for up to 24h. Total cell protein was resolved on SDS-PAGE and electrotransferred onto nitrocellulose. ECL was performed using a polyclonal human anti-caspase-3 Ab that recognizes caspase-3 (p32) and its cleavage products (p20/17). Panel A, representative filter; Panel B, densitometric analysis. Data are expressed as mean % of 6h control ± SEM; N=3 experiments.


A
Duration of culture (h)
6
12
24
-
+
-
+
-
+
32 kDa
20 kDa
17 kDa


B
150
100
50
0
control
CDDP
p32 protein content
(% of 6h control)
6
12
24
Duration of culture (h)

14.9 Antagonistic Fas Ab partially blocked apoptosis induced by CDDP.

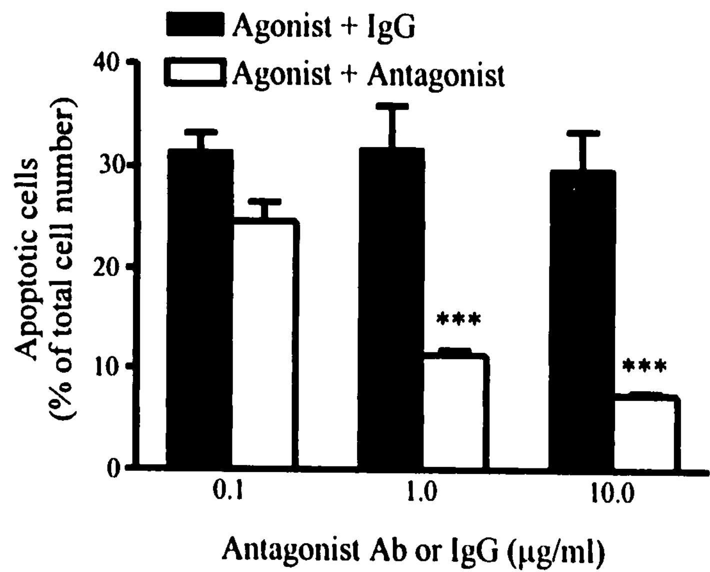
To directly answer the question of whether or not the Fas/FasL system plays a key role in CDDP-induced cell death, the influence of Fas receptor blockade with an antagonistic Fas Ab on CDDP-induced apoptosis was studied. The specificity and concentration of the Ab were determined by its ability to block apoptosis induced by a Fas agonistic Ab at a maximally effective concentration. OV2008 and C13* were preincubated with varying concentrations (0.1-10 μg/ml) of antagonistic Ab for 6 h, afterwhich agonistic Fas Ab was added for a further 24 h. The levels of apoptosis for the control groups (i.e. IgG alone and IgM plus IgG) were 3.2 % ± 0.43 and 3.4 % ± 0.45, respectively for OV20008 and 1.7% ± 0.28 and 1.98% ± 0.18, respectively for C13* (Fig.23 A and B). The presence of 1-10 μg/ml of antagonistic Fas Ab effectively blocked apoptosis induced by the agonistic Ab [2 μg/ml, previously determined to be maximal stimulatory concentration ($p<0.001$; Fig. 23 A and B)] in both OV2008 and C13*. Since the presence of 10 μg/ml of antagonistic Ab reduced apoptosis by 80% in OV2008 and C13*, this concentration was used in subsequent studies.

The cleavage of caspase-3 occurs after Fas activation and caspase-3 inhibitors prevent Fas- induced cell death (Schlegel et al 1996; Tolomeo et al 1998). In the presence of Fas agonistic Ab, caspase-3 was cleaved into its p20/17 fragment (Fig. 24) in OV2008 and C13*. This effect was blocked completely by addition of Fas antagonistic Ab, which further indicates specificity for the Fas receptor and that 10 μg/ml of antagonistic Ab was sufficient to block the receptors.

To determine if the extent to which the Fas system is involved in CDDP induced apoptosis, OV2008 cells were preincubated with antagonistic Fas Ab or IgG for 6h and subsequently cultured a further 24h in the absence or presence (10-30 μM) of CDDP with or without the addition of another 10 μg/ml of antagonistic Ab. The second addition of

Figure 23. Antagonistic Fas Ab effectively blocked apoptosis induced by agonistic Fas Ab in OV2008 and C13* hOSE cancer cells.

Varying concentrations of antagonistic Fas or IgG (0.1-10 μg/ml) were added to OV2008 (Panel A) and C13* (Panel B) cultures for 12h, afterwhich agonistic Fas Ab (2 μg/ml) was added to the culture wells for a further 24h. Control lanes were IgG alone or IgG plus IgM (not shown in figure). Apoptosis was determined by Hoechst nuclear staining as described previously. Data are expressed as mean % ± SEM; N=3 experiments; *** $p<0.001$ (compared to IgG at identical concentrations).


A
Agonist + IgG
Agonist + Antagonist
Apoptotic cells
(% of total cell number)
40
30
20
10
0
***
***
0.1
1.0
10.0
Antagonist Ab or IgG (μg/ml)

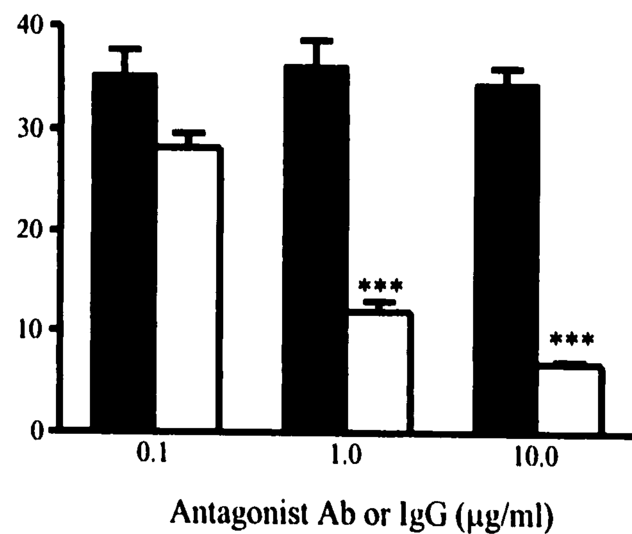
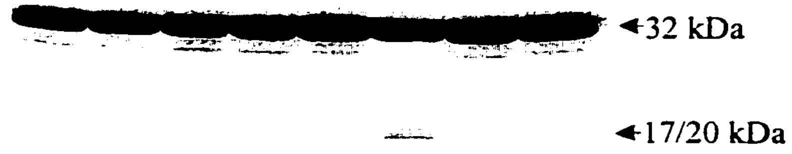
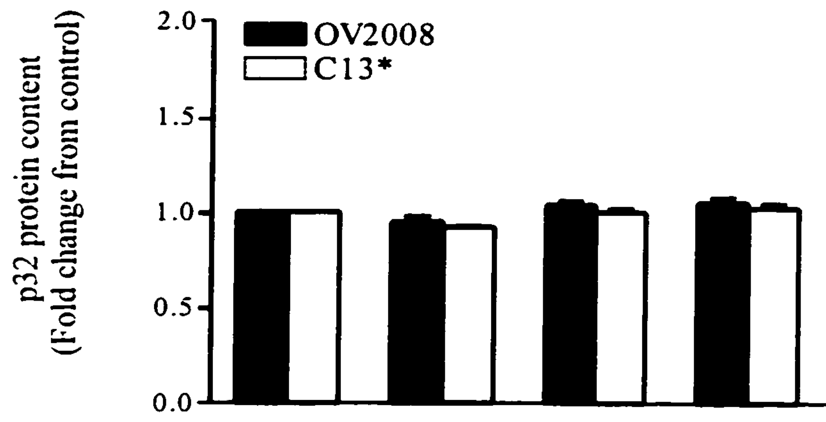

B
40
30
20
10
0
***
***
0.1
1.0
10.0
Antagonist Ab or IgG (μg/ml)

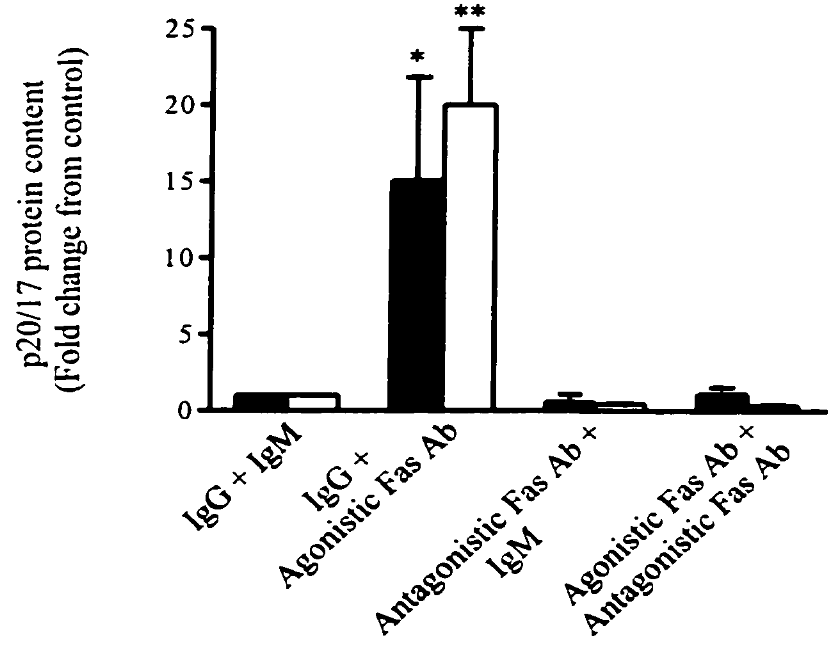
Figure 24. Antagonistic Fas Ab effectively blocked caspase-3 cleavage induced by agonistic Fas Ab in OV2008 and C13* hOSE cancer cells.

Antagonistic Fas Ab or IgG (10 μg/ml) was added to OV2008 and C13* cultures for 12h, afterwhich agonistic Fas Ab or IgM (2 μg/ml) was added to the culture wells for a further 24h. Control lanes were IgG and IgM. Proteins were extracted and resolved on SDS-PAGE. A polyclonal caspase-3 Ab was used for the Western analysis. Data are expressed as fold change from control ± SEM; N=3 experiments; * $p<0.05$, ** $p<0.01$ (compared to respective control).

	0V2008				C13*			
Antagonistic Fas Ab	-	-	+	+	-	-	+	+
Agonistic Fas Ab	-	+	-	+	-	+	-	+







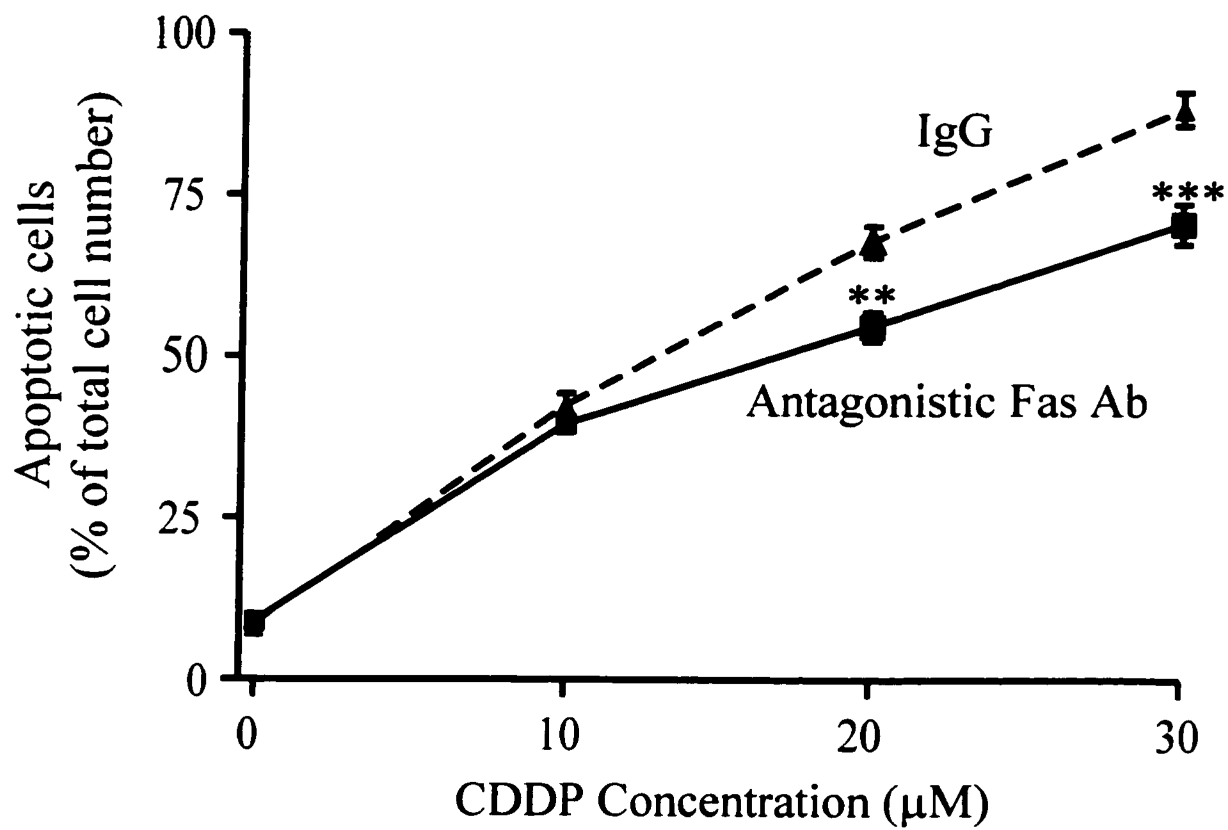
antagonistic Ab was done in order to test whether there may have been some degradation of the antibody over the 6h incubation period. In both protocols, there was a significant reduction of CDDP-induced apoptosis in the presence of the antagonistic Ab ($p<0.001$). The two protocols were statistically compared using a two way ANOVA and no significant difference between them was evident ($p>0.05$). This indicates that the addition of a second 10 µg/ml of antagonistic Ab after the 6h preincubation period had no additional effect in reducing the levels of apoptosis induced by CDDP. The data from the two protocols were pooled and are presented in Fig. 25. Blockade of the Fas receptor significantly reduced the levels of CDDP-induced apoptosis at 20 and 30 µM of CDDP ($p<0.05$ and $p<0.0001$ respectively; Fig. 25). However, the reduction in cell death was only 19.3% at 20µM CDDP and 20% at 30µM of CDDP (Fig. 25), suggesting that the involvement of Fas and FasL interaction in CDDP-induced apoptosis, may play only a relatively minor role.

14.10 The presence of Fas and FasL protein in Ovarian Carcinoma Tissue

To determine if Fas and FasL are expressed in ovarian carcinomas, Fas and FasL proteins were immunolocalized in human ovarian surface epithelial tumors using a polyclonal anti- human Fas and FasL Ab, respectively. In addition, *in situ* TUNEL and immunohistochemistry for PCNA were performed to examine if and how the expression of Fas and FasL relates to epithelial cell apoptosis and/or proliferation. PCNA positive cells were evident throughout the tumor section (Fig. 26 b; indicated in area within circle). The number of TUNEL positive cells present in the tumor was minimal (Fig. 26 a) and the expression of Fas and FasL (Fig. 26 c and d) was inversely correlated with the proliferative state of the cells. While there was a significant level of Fas in proliferatively active cells (Fig. 26c; PCNA positive; indicated in area within circle), the abundance of this cell death receptor was considerably higher in the non-

Figure 25. Blockade of the Fas Receptor partially reduced CDDP-induced apoptosis in OV2008 hOSE cancer cells.

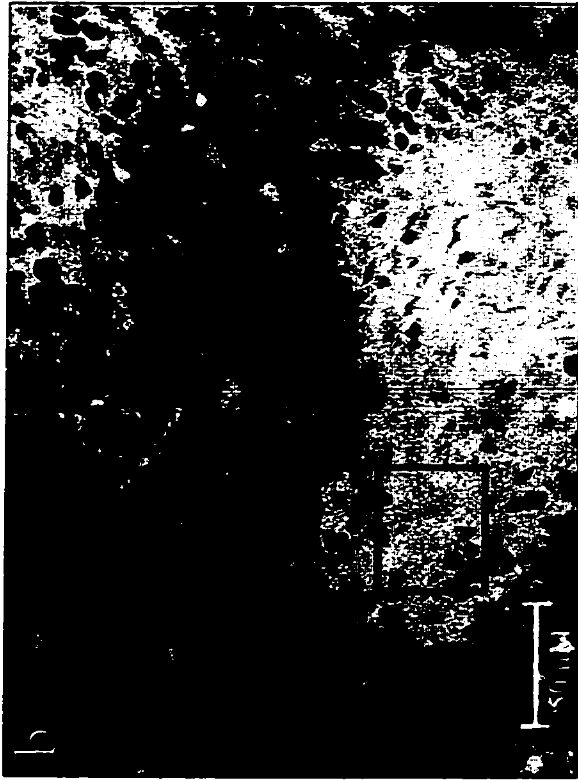
OV2008 hOSE cancer cells were preincubated with 10μg/ml of antagonistic Ab or IgG in the presence of varying concentrations of CDDP (0-30 μM) for 24h. Apoptosis was detected by Hoechst staining as described previously. Data are expressed as mean % ± SEM; N=3 experiments; ** $p<0.01$, *** $p<0.001$ (compared to respective control).

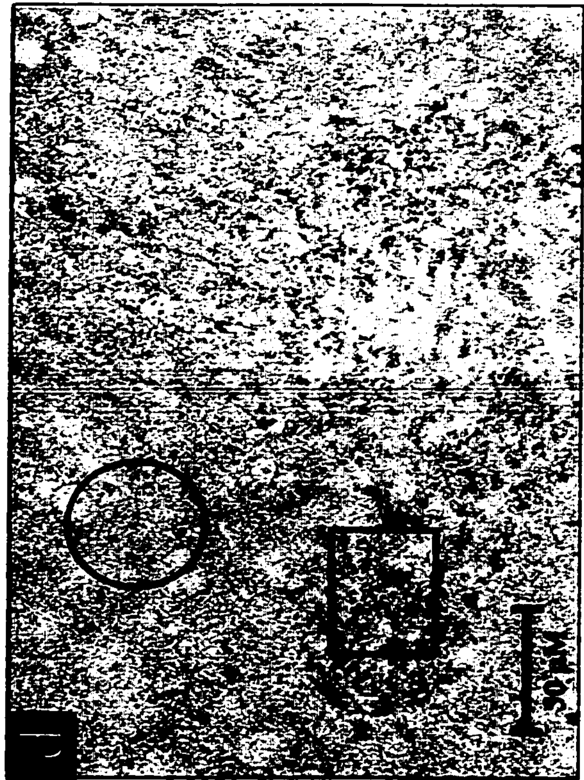


100
75
50
25
0
Apoptotic cells
(% of total cell number)
IgG
***
**
Antagonistic Fas Ab
0
10
20
30
CDDP Concentration (μM)

Figure 26. Detection of Fas/FasL, PCNA and cell death in human ovarian carcinomas.

Representative photomicrographs illustrating *in situ* detection of cell death (TUNEL; Panel a) and immunolocalization of PCNA (Panel b), Fas (Panel c) and FasL (Panel d) in human ovarian surface epithelial tumor tissue. Magnification: 400X





proliferative, non-apoptotic ones (Fig. 26c; indicated in area within square). Localization of FasL within the tumors followed a similar distribution pattern as Fas but with a relatively lower intensity compared to FasL negative regions.

15.0 DISCUSSION

15.1 Experimental Model

Ovarian cell lines have been used extensively to investigate the molecular and cellular biology of ovarian cancer cell fate and chemosensitivity (Gallion et al 1998). In this regard, OV2008 and the resistant subclone C13* have been characterized by Andrews and Howell (1990). The C13* cell line is 12-fold resistant to CDDP at the ID50. Factors responsible for this resistance include a 2-fold decrease in CDDP accumulation (Andrew and Howell, 1990), and a 4.5 fold enhancement of replicative bypass, with no difference in levels of total DNA repair activity (Mamenta et al 1994). Expression of DNA damage inducible genes gadd153, gadd45, p21 and c-jun have been demonstrated at lethal doses of CDDP in both OV2008 and C13* cells and are correlated with CDDP cytotoxcity (DelMastro et al 1997). In addition, the mitochondria appear morphologically aberrant (Andrews and Albright 1992) and have variations in protein kinase activity (Isonishi et al 1990; Isonishi et al 1992) and in cAMP signal transduction pathways (Mann et al 1991). The CDDP resistance in this cell line requires the presence of both chromosome 11 and 16 as deletion of these chromosomes resulted in the loss of the resistant phenotype (Mirakhur et al 1996). Genes for MRP, LRP, MT-II_A and ERCC4 are present on chromosome 16. OV2008 and C13* show no discernble differences in MRP gene expression (Parekh and Simpkins 1996) and the roles of LRP, MT-II_A and ERCC4 have not been determined. Although GST-π and SSRP (two genes on chromosome 11) have been implicated in CDDP resistance, GST-π does not appear to play a role in the resistance in the C13* cells (Mirakhur et al 1996) and glutathione levels are not significantly different between these sensitive OV2008 and resistant C13* cells (Parekh and Simpkins 1996). The function of SSRP in OV2008 and C13*cells is not known.

The A2780-cp cell line is 5-fold resistant to CDDP at the IC50 (38.5 μM) (Masuda et al 1990). Factors responsible for this resistance include a 2-fold increase in repairing CDDP lesions than the sensitive parent, A2780-s (Masuda et al 1990). In addition, A2780-cp cells contains elevated GSH levels (Louie et al 1985) and carry a nonfunctional mutant p53 (Skilling et al 1996). The roles of MRP, LRP, $MT\text{-}II_A$, ERCC4, and SSRP in these cell lines have not been determined.

In the present studies, I have exploited the availability of two pairs of CDDP-sensitive (OV2008 and A2780-s) and -resistant (C13* and A2780-cp, respectively) hOSE cancer cell lines to investigate the role and regulation of the Fas/FasL system in the control of apoptosis and chemoresistance. I have demonstrated that the expression of Fas is upregulated by CDDP *in vitro* in both the sensitive (OV2008 and A2780-s) and one resistant variant (C13*) cell line. FasL upregulation is characteristic of the CDDP-sensitive cells but not of resistant variants (C13* and A2780-cp), suggesting a possible role of this death ligand in chemoresistance. Cell lines are convenient, readily available and amenible to manipulations. However, a concern in using cell lines is that the cells might have lost their original cellular characteristics with increased passages. For this reason, future studies with primary cultures should be carried out to ascertain that the responses observed with the cell lines are indeed reflective of those noted in primary cultures. Ideally, *in vivo* surveys of the cellular and molecular markers should be performed to determine the effect of CDDP on tumor progression and chemosensitivity. During surgical debulking, tumors from patients prior to and following CDDP treatment should be retreived for further studies. In this context, this prelimanary study has demonstrated the presence of Fas and FasL protein in human ovarian carcinomas, suggesting at least the presence of these proteins in these cell lines may be physiologically significant.

15.2 The influence of CDDP on Fas and FasL expression and apoptosis on Ovarian cancer cells.

In the present studies, I have demonstrated that CDDP induces apoptosis in the sensitive OV2008 and A2780-s cells in a concentration- and time-dependent manner. There were no significant decreases in cell number during CDDP treatment except at a high concentration (30μM). It has been demonstrated that CDDP can induce necrosis in addition to apoptosis at high concentrations (Lieberthal et al 1996). In certain circumstances, loss of plasma membrane integrity can be a late feature of apoptosis. This process, called 'apoptosis necrosis', is usually seen whenever apoptotic cells escape phagocytosis. This can occur *in vivo*, but is more commonly seen *in vitro* because of the absence of phagocytic cells (Lieberthal et al 1996). Guchelaar et al (1998) demonstrated that CDDP induced apoptosis in human leukemia cells and that necrosis also occured but through an indirect pathway following the apoptotic state. Thus, the cell loss observed at high concentrations of CDDP (30 μM) in this present study is most likely due to secondary necrosis. There was no significant cell loss at lower concentrations, thus the percentage of cell death measured was probably due to apoptosis and not necrosis.

Several DNA damaging agents, including CDDP, have been shown to upregulate Fas and FasL expression in a variety of tumour cell types including ovarian cancer cells (Friesen et al 1996; Uslu et al 1996; MÜller et al 1997; Fuchs et al 1997; Villunger et al 1997; Tolomeo et al 1998; Kasibhatla et al 1998). In the present studies, I have demonstrated that the presence of significant levels of Fas and FasL in both OV2008 and A2780-s cells, and that CDDP treatment resulted in upregulation of these proteins, raising the possibility of a paracrine role for FasL in CDDP-induced cell death.

Recent reports have demonstrated a soluble FasL (26 kDa) as a cleavage product of a cell-associated FasL via the action of a metalloproteinase (Tanaka et al 1998). I was unable to detect a 26kDa soluble FasL in cultures of our four ovarian cancer cell lines,

irrespective of the CDDP concentration and the duration of cultures. Instead, this study demonstrates the presence of an immunoreactive FasL in the spent media of ovarian cancer cell cultures with similar molecular size (37kDa) as that of the cell-associated protein. While differences in cell type and/or glycosylation patterns could have contributed to the large variations in the molecular size of FasL reported (Suda and Nagata 1994; Tanaka et al 1995), it is unlikely that, based on it molecular size, the immunoreactive soluble FasL in the present studies is a cleaved form of the cell associated protein. While the mechanism of release of this membrane protein after synthesis is unclear, its presence in the spent media could not be attributed to the presence of cellular debris containing FasL, as (a) its level was unaffected by attempts to further purify the media by ultracentrifugation, (b) the lack of an increase in an immunoreactive membrane protein (e.g. Fas) in the media even with increasing concentration of CDDP, and (c) it was cell type-specific (sensitive but not resistant cells).

The activation of caspases, specifically of caspase-8 and -3 in Fas-mediated and of caspase-3 in drug-induced apoptosis, has been demonstrated in many cell types (Friesen et al 1997; Fulda et al 1997; Fuchs et al 1997; Gamen et al 1997; Eischen et al 1997). Some authors report that specific inhibitors of caspase-3 can inhibit Fas-, irradiation- and drug- induced apoptosis (Fuchs et al 1997), or just nuclear apoptosis (Gamen et al 1997). Others, however, reported that while general caspase inhibitors can block drug-mediated apoptosis, specific caspase-3 inhibitors have little effect on the induction of apoptosis (Tolomeo et al 1998). It seems that caspases are essential for drug-induced apoptosis, but no one caspase is crucial as redundancy in the caspase system exists. As a result, one inactivating mutation in a caspase may not necessarily lead to resistance to drug-induced apoptosis. Although inhibition of caspase-3 blocks Fas-mediated apoptosis in many cell types (Tolmeo et al 1998), Fas apoptosis pathway was not affected in most tissues in caspase-3 deficient mice, indicating that the requirement of caspase-3 may be cell type specific (Kuida et al 1996). In the present

study, CDDP caused the cleavage of caspase-3 and -8 into their active fragments in OV2008 cells. Fas activation results in the cleavage of caspase-3 and presumably also of caspase-8 in both cell types, since the latter is required for Fas-mediated apoptosis. Time course analysis of caspase-3 and -8 cleavage reveal that their activation occurs approximately at 12h. It is difficult to assess whether caspase-8 activation occurs before that of caspase-3, which would suggest that CDDP activation of caspase-3 proceeds through receptor mediated events. These findings are consistent with the notion that the increased expression of Fas and FasL following CDDP is functional and downstream caspases are activated. The cleavage of caspase-8 after CDDP exposure is intriguing, since this protease is only known to be activated by Fas, TNF or DR3 receptors. Since membrane association and oligomerization of caspase-8 are essential for its activation (Martin et al 1998), the activation of caspase-8 by CDDP suggests a role for receptor mediated apoptosis in CDDP cytotoxicity.

15.3 Possible Role of the Fas/FasL system in CDDP-induced apoptosis in hOSE cancer cells.

Considerable controversy exists over the role of the Fas/FasL system in drug-induced apoptosis. Fas-resistant T-cell leukemia and neuroblastoma were cross-resistant to various drugs including CDDP (Cai et al 1996; Friesen et al 1996; Fulda et al 1997). Blockade of the Fas receptor in T-cell leukemia, neuroblastoma, hepatoma cells and some solid tumors attenuated DXR-, methotrexate- and CDDP-induced apoptosis (Friesen et al 1996; Müller et al 1996; Friesen et al 1997; Fulda et al 1998). Inhibition of NF-κB activation and hence FasL expression in a Jurkat cells blocked apoptosis induced by etoposide and teniposide (Kasibhatla et al 1998). However, others have found that inhibiting Fas-induced apoptosis in T-cell leukemic cell lines did not cause resistance to DNA-damaging agents (Gamen et al 1997; Eischen et al 1997; Tolomeo et al 1998). Fas-

deficient thymocytes were still sensitive to DXR (Fuchs et al 1997) and blocking the Fas pathway did not antagonize CEM cells to a variety of drugs including CDDP (Villunger et al 1997). FADD-deficient cells were sensitive to adriamycin as were their wild-type counterparts (Yeh et al 1998). In terms of ovarian cancer, cells (SKOV-3 and SiHa) expressing the Fas receptor but resistant to agonistic Ab activation remain sensitive to CDDP (Wakahara et al 1997), raising the possibility that the Fas signaling death pathway may play a minimal role in CDDP-mediate apoptosis.

In the present studies, in addition to the increase in Fas and FasL expression in sensitive ovarian cancer cells in response to CDDP, Fas activation by exogenous agonistic Fas Ab alone leads to apoptosis, confirming that the receptor and post-receptor signaling pathway are functional. Moreover, treatment of OV2008 cells with CDDP markedly increased their responsiveness to agonistic Fas Ab in the induction of apoptosis. These findings are consistent with our observation that CDDP upregulates the expression of functional Fas in these cells, and that these two agents are acting via different but complementary mechanisms (i.e. induction vs activation of Fas). Consistent with our findings are the reports that the cytocidal effects of the Fas Ab and adriamycin or CDDP are synergistic in a number of cell types (Wakahara et al 1997; Uslu et al 1996; Morimoto et al 1993).

This study also demonstrated that addition of spent media to ovarian cancer cell cultures significantly increased apoptosis which could be attenuated by pretreatment with antagonistic Fas Ab. Furthermore, addition of spent media to cells in the presence of maximal stimulatory concentration of agonistic Fas Ab failed to further increase apoptosis induced by the Fas agonist, suggesting that the active element(s) in the spent medium is functional and can activate the same death pathway as agonistic Fas Ab. Our observations differ from the recent studies of Tanaka et al (1998), which showed low activity of sFasL compared to its membrane bound form and suggested an antagonistic action of this soluble protein on the cytotoxic effects of membrane bound FasL. However,

in cell types that express ample amount of Fas, human sFasL alone is able to induce apoptosis and does not effectively inhibit cell death induced by membrane bound FasL on neighbouring cells (Perez et al 1990).

It has been suggested that mFasL is more potent because it causes prolonged crosslinking of Fas, which can activate the apoptotic death pathway. Complexes of Fas and sFasL, however, can be rapidly internalized, and this may prevent formation of the death-inducing signaling complex. It is also possible that apoptosis induced by sFas may have to be assessed at a longer time point. Although the presence of sFasL in the supernatant was not determined, the ability of the antagonistic Fas Ab to only partially inhibit apoptosis induced by the spent media, as observed in the present studies, suggest that other apoptosis-inducing factors might have been present.

It is of interest to note from the present studies that blockade of the Fas receptor in OV2008 cells with an antagonistic Fas Ab attenuated CDDP-induced apoptosis by 20 %, revealing that the Fas system may have a relatively minor role in the induction of apoptosis by this chemotherapeutic agent in this cell type. It seems that other mechanisms may be involved, including those of other death receptors (i.e. DR5). In this context, it has been demonstrated that DXR induces DR5 gene expression and apoptosis in breast cancer cells (Sheikh et al 1998). Non-receptor signaling may mediate CDDP-induced apoptosis. It has been demonstrated that the IAP family modulates apoptotic signalling by regulating caspase cleavage and activation (Deveraux et al 1997). In addition, studies from our laboratory have demonstrated that downregulation of XIAP with adenoviral antisense cDNA alone resulted in apoptosis (Tsang et al 1999). Thus direct modulation of caspases may be important in CDDP cytotoxicity. The relative importance of the Fas/ FasL system in the cell death process may be dependent on the tumor type and cytotoxic agent in question.

15.4 A Possible Role of the Fas/FasL System in Chemoresistance.

While the success of genotoxic anticancer therapy has been shown to be dependent on effective activation of apoptosis in tumor cells in response to DNA damage, genotypic alterations in human cancers that interfere with DNA damage-induced apoptosis are believed to confer resistance to chemotherapeutic agents or irradiation (Perego et al 1996). In the present studies, the concentrations of CDDP tested failed to induce apoptosis in the resistant (C13*) cells, although 30 μM of CDDP induced apoptosis minimally in the resistant (A2780-cp) variant. The C13* cells are known to exhibit a CDDP accumulation defect approximately 2-3 fold relative to OV2008 (Zinkewich-Peotti and Andrews, 1992) and an enhanced replicative bypass (Mamenta et al 1994). Our current findings indicate an ED50 of 10μM for the induction of apoptosis in OV2008 cells. Thus, considering the difference in CDDP accumulation, the presence of 30μM CDDP in the resistant cells would have provided equivalent cellular concentration of the agent necessary to induce apoptosis by >50%. In addition, the reported ID50 for C13* is 37μM [based on standard growth inhibition assays (Delmastro et al 1997)], suggesting that at 30μM, enough CDDP was present to cause DNA damage and inhibit growth.

I have demonstrated that both OV2008 and C13* cells contained similar basal levels of Fas protein, and that Fas contents increased in response to CDDP in both cell types, suggesting that CDDP resistance could not be due to lack of its expression in C13* cells. In addition, Fas activation by exogenous agonistic Fas Ab leads to apoptosis in both CDDP-sensitive and -resistant cells, although not to the same extent as that observed in the presence of CDDP, confirming that the receptor and post-receptor signaling pathway is functional but suboptimal, and that defects may, in part, be proximal to Fas. The present studies have shown that Fas increased transiently in the C13* cells, with levels returning to control levels at the highest CDDP concentration tested. It is possible

that the cells initially responded to CDDP with an increase in Fas, and with time and higher concentrations, other mechanisms (e.g. Fas internalization) might be involved in bringing CDDP-induced Fas protein content back to control levels and decreasing the impact of CDDP. Recent studies by Uslu et al (1996) demonstrate that sub-toxic levels of CDDP are capable of inducing Fas expression in the CDDP-resistant ovarian cancer cell line (A2780-cp70), however Friesen et al (1997) reported that CDDP resistant neuroblastoma cell line failed to respond to CDDP in terms of Fas expression, suggesting that CDDP upregulation of Fas may be cell type specific.

Unlike their CDDP-sensitive cells, the resistant counterparts (C13* and A2780-cp), while containing significant basal levels of FasL, failed to respond to the chemotherapeutic agent in the expression of this protein, and to undergo apoptosis *in vitro*. Although the actual increase in cell-associated FasL content in the sensitive hOSE cancer cells (OV2008 and A2780-s) was minimal, that of the sFasL in the OV2008 cultures was almost 3-4 fold higher in the presence of 30 μM CDDP. In contrast, the levels of sFasL in C13* cell cultures were only about 20% of those detected in the spent media of OV2008 cells. Friesen et al (1997) also reported that doxorubicin (DXR) resistant hepatoma cells failed to upregulate FasL in response to DXR, and cell surface Fas expression was absent. In addition, a sub-line of Jurkat cells that developed resistance in Fas- and TNF-induced apoptosis, also developed resistance to doxorubicin. There was a complete loss of Fas and TNFR surface expression as well as caspase-3 expression (Martinez-Lorenzo et al 1998). In the present study, CDDP treatment of resistant hOSE cancer cells (C13*) did not result in the cleavage of caspase-8 and -3 into their active fragments as seen in OV2008 cells. Fas activation results in the cleavage of caspase-3 in both cell types, since the latter is required for Fas-mediated apoptosis. In this context, the absence of drug-induced FasL and caspase activity have recently been reported in drug resistant solid tumors (Fulda et al 1998). These findings suggest that failure to express

adequate FasL in response to chemotherapeutic agents (e.g DXR and CDDP) may contribute to their chemoresistance.

The p53 tumour suppresser gene is believed to be an important component of DNA damage-induced apoptosis in many cancer cells and mutation of this gene is believed to be associated with chemoresistance. Based on the ability of both OV2008 and C13* to express p53 responsive genes (thus indicative of the presence of a wild type p53; DelMastro et al 1997), it is unlikely that the inability to express FasL and chemoresistance noted in the C13* cells is a result of a defective p53. In contrast, whereas Fas and FasL expression in A2780-s were upregulated by CDDP *in vitro*, the expression of these cell death proteins in the resistant variant (A2780cp) was not affected by the chemotherapeutic agent. Since the CDDP-resistant A2780-cp hOSE cancer cells, but not its sensitive counterpart (A2780-s), have a mutated p53 (Skilling et al 1996), the possibility of a role of mutated p53 in the regulation of these proteins cannot be excluded. In addition, these results demonstrate that p53 may be required for Fas but not FasL upregulation. These observations are consistent with earlier findings that upregulation of Fas in hepatoma, non-small-cell lung adenocarcinoma and erythraleukemia cells in response to a DNA-damaging agent, such as CDDP, is p53-dependent (Fuchs et al 1997; Owen-Schaub et al 1995), whereas FasL regulation is not dependent on p53 status (Müller et al 1997; Tolomeo et al 1998). In addition, studies from our laboratory have demonstrated that overexpression of p53 in rat granulosa cells upregulated Fas but not FasL expression and induced apoptosis which could be augmented by exogenous agonistic Fas Ab (Kim et al 1999). Although there is no p53 consensus sequence in the upstream region of Fas, these data strongly support the hypothesis that p53 may play a key role in mediatng CDDP induced Fas expression hOSE cancer cells. In spite of the above discussion, it should be noted that not all drug-mediated apoptosis is p53-dependent (Tolomeo et al 1998). On the other hand, current evidence also supports the notion that FasL expression can be regulated by the transcription factors NF-κB and AP-1

which are activated in response to a variety of drugs (Kasibhatla et al 1998). Reactive oxygen species (ROS), which are generated by some chemotherapeutic agents, can regulate NF-κB and in turn FasL expression (Bauer et al 1998).

Dysregulation of the Fas and FasL cell death system has been suggested to contribute to chemoresistance (Cai et al 1996). However, blockade of the Fas receptor in OV2008 with an antagonistic Fas Ab but minimally (~20%) abbrogated CDDP-induced apoptosis. This suggests that involvment of Fas/FasL in the cytotoxic action of CDDP may be relatively minor. Thus, despite the apparent failure of CDDP to upregulate FasL and sFasL in resistant cells (C13*), it is possible they are not major contributors to the chemoresistance phenotype. In fact, use of receptor events to mediate the apoptotic response to DNA damage seems to be an inefficient and roundabout mechanism. However, upregulation of Fas and FasL in response to various cytotoxic agents is commonly observed. It may be that the regulation of cell death via transcriptional control is desirable to allow several checks and balances on the system. In addition, use of a cell surface receptor/ligand pair to activate caspases might provide an additional fail-safe mechanism to ensure that death occurs. Since cells that undergo cytotoxic stress are likely to be in the environment of other cells facing the same stress, a cell that might avoid undergoing apoptosis through various means may nevertheless undergo apoptosis through interaction with a FasL expressing neighbor. In this way, possible escape from DNA damage-induced apoptosis might be minimized.

Apoptosis induced via Fas is mediated primarily by caspase-8 activation of downstream caspases and mitochondrial factors, though not essential, are believed to amplify this pathway (Scaffidi et al 1998). This is consistent with the observation that *Bcl-2* is frequently not effective in blocking Fas-induced apoptosis. *Bcl-2* expression can effectively block CDDP-induced apoptosis in glioma, breast cancer and myeloid leukemia cells (Weller et al 1995), suggesting a critical involvement of mitochondrial factors (e.g. cyt c and caspase-9) in drug-induced apoptosis. In fact, inactivation of

caspase-9 and Apaf-1 disrupts p53-dependent apoptosis (Sorengo et al 1999). It is not surprising that high levels of *Bcl-2* can confer resistance to chemotherapeutic agents and several resistant cells are known to overexpress *Bcl-2* (Eliopoulos et al 1995). Fewer studies have focused on apoptotic genes outside the p53 and *Bcl-2* family. Activation of caspases can occur by downregulation of IAPs in both OV2008 and C13* (Tsang et al 1999). More importantly, CDDP inhibited IAP expression and induced apoptosis in sensitive but not resistant cells. Whereas CDDP alone failed to induce apoptosis in the resistant cells, treatment of these cells with the chemotherapeutic agent significantly augmented cell kill brought about by agonistic Fas Ab. This increase in cell death may be a result of increased cell surface Fas expression brought about by the CDDP treatment. In contrast to the CDDP-sensitive (OV2008) cells, there was a lack of synergy with the combination of agonistic Fas Ab and CDDP treatment. This could be partly explained by studies from our laboratory demonstrating that CDDP can downregulate XIAP expression, thus allowing derepression of caspase-3 activity in OV2008 cells but not C13* cells (Tsang et al 1999). This could account for the synergistic effect of CDDP and agonistic Fas Ab on apoptosis in OV2008 cells but not C13* cells. In support of this, overexpression of XIAP attenuated CDDP induced apoptosis, implicating XIAP as a potential contributing factor in chemoresistance. It is likely that many redundant pathways are intiated in response to DNA damage to ensure complete cell destruction and avoid neoplastic transformation.

15.5 Conclusions

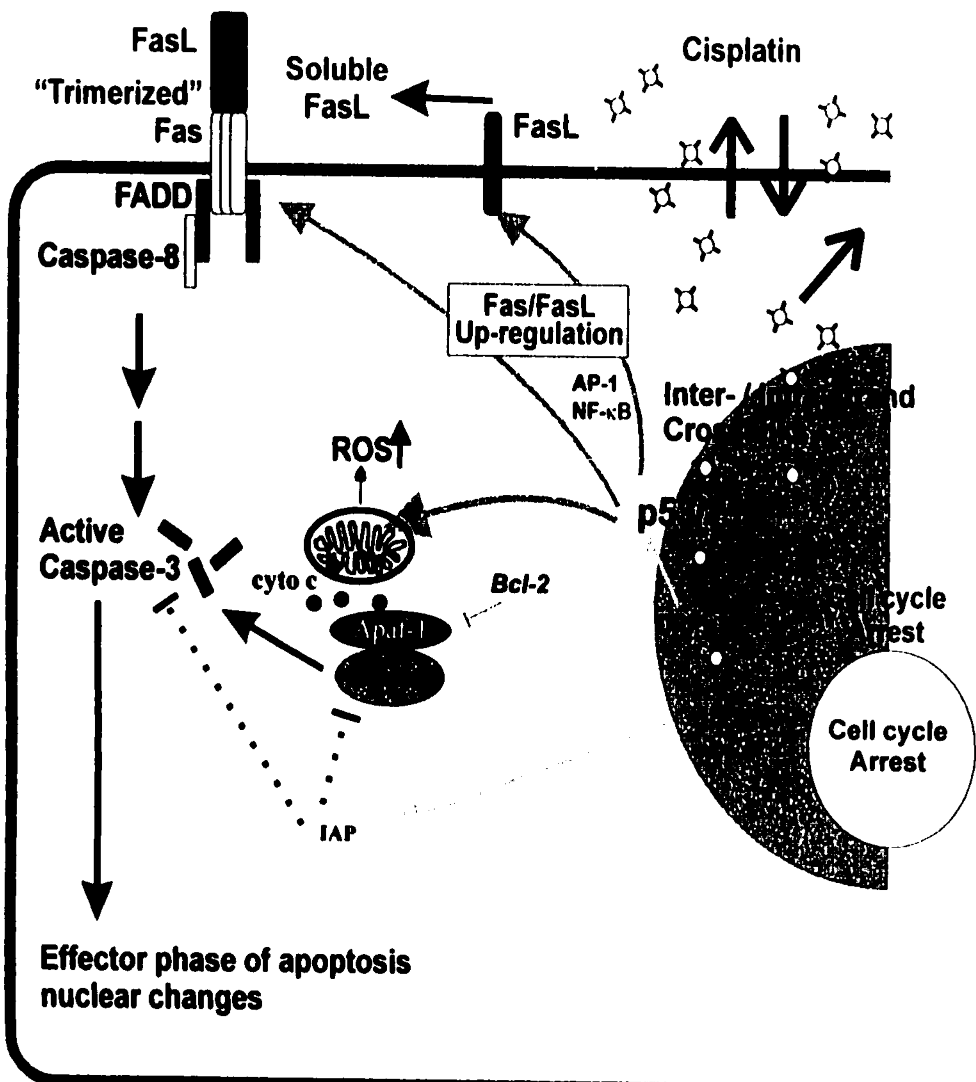
The model depicted in Figure 27 demonstrates that p53 (or some other factor) is activated following CDDP-induced DNA damage and, through a yet unknown mechanism, Fas expression is induced. FasL expression proceeds via NF-κB and/or AP-1 through ROS and/or the JNK pathway. Activation of the caspases can be through

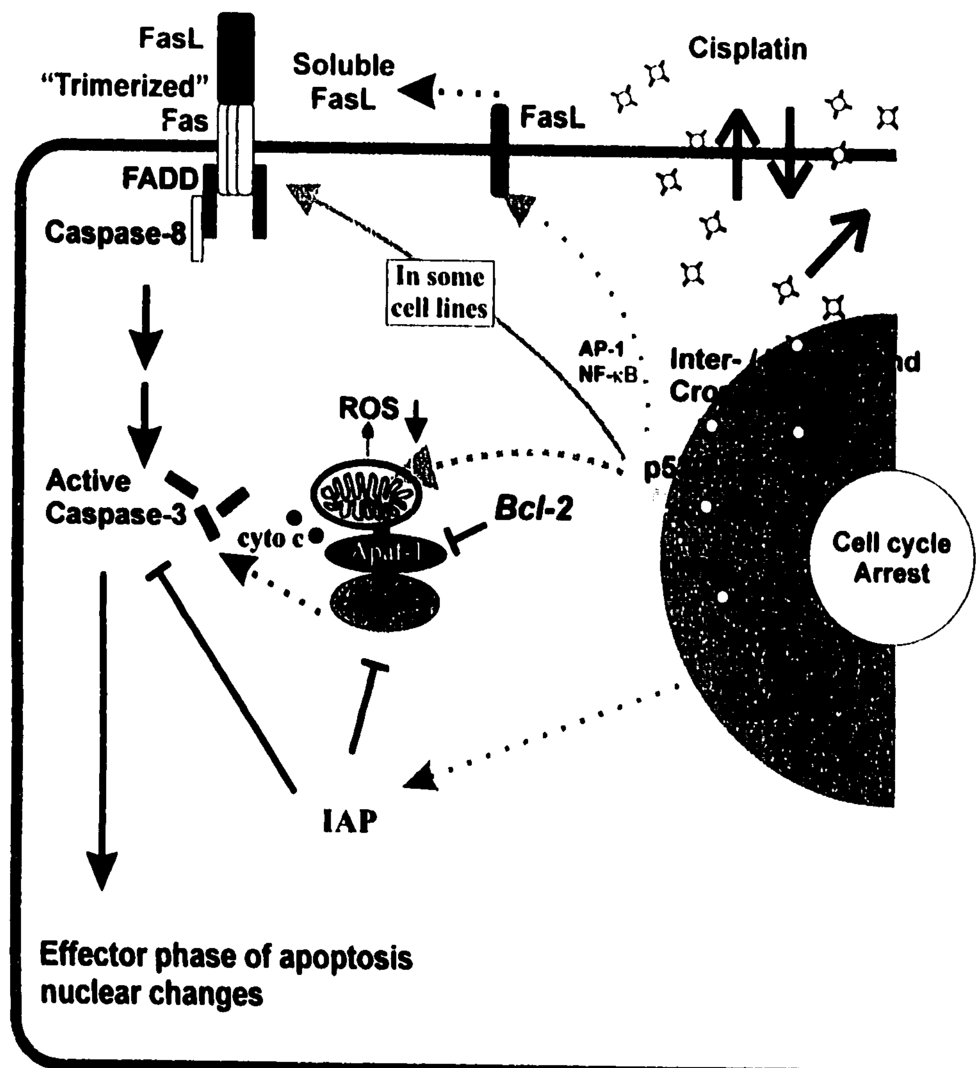
receptor mediated signaling or by direct activation of mitochondrial factors. In fact, cyto c release in drug-induced apoptosis may be caspase and receptor independent since a general caspase inhibitor was able to block *Bid* cleavage by upstream caspases, but not the release of cyt c in etoposide treated Jurkat cells (Sun et al 1999).

Since Fas agonistic Abs can enhance CDDP-induced apoptosis in OV2008 and C13*, as well as other hOSE cancer cells (Wakahara et al 1996; Uslu et al 1996), it may be of benefit in the treatment of chemo-sensitive and -resistant ovarian cancers. The presence of Fas and FasL in fresh human ovarian epithelial carcinomas clearly demonstrates that these factors are not confined to the tissue culture conditions and may be of clinical relevance. Localization of high levels of Fas in mainly non-proliferative cells which were not apoptotic is intriguing and may represent a group of cells that are waiting for an appropriate signal to activate cell death. FasL exhibited a similar distribution pattern as Fas within the tumour and, in general, was in low abundance. While Fas expression has been demonstrated in a variety of human tissues, the ability of agonist Fas mAb to activate Fas and induce apoptosis *in vivo* remains to be demonstrated. However, intra-abdominal injection of FasL, reduced tumours in mice without systemic toxicity (Rensing-Ehl et al 1995). Of all the Fas expressing tissues, the liver seems the most susceptible, and agonistic Fas Ab injected into mice has caused extensive liver destruction (Ogasawara et al 1993). Therefore to be effective in therapy, concentrations of FasL that are effective yet not toxic would be used. As CDDP is known to be highly toxic, it is tempting to suggest that a combination therapy involving these two agents may allow maximal therapeutic efficiency with overall reduction in toxicity in the treatment of CDDP- sensitive tumors. In addition, our *in vitro* data from the C13* cells also raise

Figure 27. Model

Chemo-sensitive cells detect CDDP induced DNA damage by way of damage recognition proteins (DRPs). In turn the p53 protein accumulates and can activate a number of factors involved in cell cycle arrest and induction of apoptosis. Upregulation of Fas, FasL and sFasL and subsequent activation of caspases can aid in CDDP induced cell death. Upregulation of FasL can occur via the transcription factors NF-κB and/or AP-1. In addition, non-receptor activation of caspase-3 can occur by downregulation of XIAP and/or activation of mitochondrial factors (caspase-9 and Apaf-1 complex). Chemo-resistant cells are often defective in p53 accumulation in response to DNA damage. This can result in the absence of Fas receptor upregulation. In addition, these cells can be defective in FasL and sFasL upregulation attenuating CDDP-induced apoptosis. Lack of XIAP downregulation and inhibition of caspase-9 activation by *Bcl-2* can all suppress the activation of the effector caspase-3.





Key: Proteolytic cleavage = Transport = Increased content
= Gene regulation = Inhibition = Decreased content
= Inhibition/absence

the question of a possible application of combined immunotherapy and chemotherapy (i.e. Fas mAb plus CDDP) in the management of CDDP-resistant ovarian tumors, either alone or as an adjunct to surgical reduction.

In summary, I have surveyed two sets of CDDP-sensitive and -resistant ovarian epithelial cancer cell lines and have compared their responsiveness to CDDP in terms of Fas, FasL and caspase expression as well as apoptosis. While chemoresistance in one cell line appeared to be associated with its inability to upregulate FasL, defective expression of both Fas and FasL was evident in the other resistant cell line. Although the mechanism of chemoresistance in human ovarian cancer is unknown and may be multifactorial, the present studies demonstrate that inadequate expression of FasL and sFasL may attenuate the ability of the cells to undergo apoptosis in response to chemotherapeutic agents. However, this study raises the possibility of exploiting the Fas system as a potential target for adjunct therapy in the treatment of ovarian epithelial cancer. More studies are required to determine if this concept is applicable to the *in vivo* situation.

16.0 REFERENCES

Abreu-Martin MT, Vidrich A, Lynch DH, Targan SR. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-α and ligation of Fas antigen. J Immunol 15:4147-4154, 1995.

Adams JM, Cory S. The Bcl2 protein family: Arbiters of cell survival. Science 281: 1322-1326, 1998.

Aebi S. Kurdihaidar B, Gordon R, Cenni B, Zheng H, Fink D, Christen RD, Bolaand CR, Koi M, Fishel R, Howell SB. Loan of DNA mismatch repair in acquired resistance to cisplatin. Cancer Res 56:3087-3090, 1996.

Afanas'ev VN, Korol BA, Mantsygin YA, Nelipovich PA Pechatnidov VA, Umansky SR Flow cytometry and biochemical analysis of DNA degradation characteristic of two types of cell death. FEBS Lett 194:347-350, 1986.

Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, *survivin*, expressed in cancer and lymphoma. Nature Medicine 3:917- 921, 1997.

Andrews PA, Albright KD. Mitochondrial defects in cis-diamminedichlororplatinum (II)-resistant human ovarian carcinoma cells. Cancer Res 52:1895-1901, 1992.

Andrews PA, Howell SB. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cells 2: 351, 1990.

Andrews PA, Murphy M, Howell SB. Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. Cancer Res 45: 6250-6253, 1985.

Andrews PA, Murphy M, Howell SB. Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. Cancer Chemother Pharmacol 19:149-154 1987.

Anthoney DA, McIlwrath AJ, Gallagher WM, Edlin AR, Brown R. Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumour cells. Cancer Res 56: 1374-1387, 1996.

Asahina H, Kuraoka I, Shirakawa M, Morita EH, Miura N, Miyamoto I, Ohtsuka E, Okada Y, Tanaka K. The XPA protein is a zinc metalloprotein with an ability to recognize various kinds of DNA damage. Mutat Res 315:229-237, 1994.

Auersperg N, MacLaren IA, Kruk PA. Ovarian Surface Epithelium: Autonomous Production of Connective Tissue-Type Extracellular Matrix. Biol of Repro 44:717-724, 1991.

Auersperg N, Maines-Bandiera SL, Dyck, HG, Kruk PA. Characterization of cultured human ovarian surface epithelial cells: Phenotype plasticity and premalignant changes. Lab Invest 71:510-518, 1994.

Auersperg N, Maines-Bandiera SL, Dyck H. Ovarian Carcinogenesis and the Biology of Ovarian Surface Epithelium. J Cell Phys 173:261-265, 1997.

Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC. The Biology of Ovarian Cancer. Sem Onc 25:281-304, 1998.

Ashkenazi A, Dixit VM. Death Receptor: Signaling and Modulation. Science 281: 1305-1308, 1998.

Baiocchi G, Kavanagh JJ, Talpaz M, Wharton JT, Gutterman JU, Kurzrock R. Expression of the macrophage colony stimulating factor and its receptor in gynecologic malignancies. Cancer 67:990-996 , 1991.

Baker V, Borst MP, Dixon , Hatch KD, Shingleton HM, Miller D. c-myc amplification in ovarian cancer. Gynecol Oncol 38:340-342, 1990.

Bast RC, Boyer CM, Jacobs I, Xu FJ Wu S, Weiner J, Kohler M. Cell growth regulation in epithelial ovarian cancer. Cancer 71:1597-1601, 1993.

Bauer MKA, Vogt M, Los M, Siegel J, Wesselborg S, Schulze-Osthoff K. Role of Reactive Oxygen Intermediates in Activation-induced CD95 (APO-1/Fas) Ligand Expression. J Biol Chem 273:8048-8055, 1998.

Bennett M, Macdonald K, Chan S-W, Luzio JP, Simari R Weissberg P. Cell Surface Trafficking of Fas: A Rapid Mechanism of p53-Mediated Apoptosis. Science 282:290-293, 1998.

Berchuck A, Kohler MF, Boente MP, Rodriguez GC, Whitaker RS, Bast RC. Growth regulation and transformation of ovarian epithelium. Cancer 71:545-551, 1993.

Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, Clarke-Pearson DL, Bast RC Jr. Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-β. Am J Obst Gyn 166:676-684, 1992.

Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB. CD28 co-stimulation can promote T cell survival by enhancing the expression of Bcl-xl. Immunity 3:87-98, 1995.

Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD- interacting protease, in Fas/APO-1 and TNF receptor- induced cell death. Cell 85:803-815, 1996.

Boone DL, Tsang BK. Identification and Localization of Deoxyribonuclease I in the Rat Ovary. Biol Repro 57:813-821, 1997.

Bourhis J, Goldstein LJ, Riou G, Pastan I, Gottesmman MM, Benard J. Expression of a human multidrug resistance gene in ovarian carcinomas. Cancer Res 49:5062-5065, 1989.

Bruhn SL, Pil PM, Essigmann JM, Housman DE, Lippard SJ. Isolation and characterization of human cDNA clones encoding a high mobility group box protein that recognizes structural distortions to DNA caused by binding of the anticancer agent cisplatin. Proc Natl Acad Sci USA 89:2307-2311, 1992.

Cai Z, Stancou R, Korner M, Chouaib S. Impairment of Fas Antigen expression in adriamycin-resistant but not TNF-resistant MCF7 tumour cells. Int J Cancer 68: 535-546, 1996.

Cascino I, Fiucci G, Papoff G, Ruberti G. Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. J Immunol 154:1157-1163, 1995.

Chapman RS, Whetton AD, Chresta CM, Dive C. Characterization of drug resistance mediated via the suppression of apoptosis by Abelson protein tyrosine kinase. Mol Pharmacol 48:334-343, 1995.

Cheng JQ, Godwin AK, Bellacosa A, Tagucki T, Franke TF, Hamilton TC, Tsichlis PN, Testa FR. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci, USA 89:9267-9271, 1992.

Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Keifer MC, Barr PJ, Mountz JD. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science 263:1759-1762, 1994.

Chow CS, Whitehead JP, Lippard SJ. HMG domain proteins induce sharp bends in cisplatin-modified DNA. Biochemistry 33:15124-15130, 1994.

Christofori G, Naik P, Hanahan A. Second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. Nature 369:414-418, 1994.

Chu G, Chang E. Cisplatin-resistant cells express increased levels of a factor that recognizes damaged DNA. Proc Natl Acad Sci USA 87:3324-3327, 1990.

Cifone MG, DeMaria R, Roncaioli P, Rippo MR, Azuma M Lanier LL, Santoni A, Testi R. Apoptotic signaling through CD95 (Fas/Apo1) activates an acidic sphingomyelinase. J Exp Med 180: 1547-1552, 1994.

Cole SP, Bharduaji GG, Gerlach JM, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kruz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a multi-drug resistant human lung cancer cell line. Science 258:1650-1654, 1992.

Cotter TG, Lennon SV, Glynn JM, Green DR. Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis. Cancer Res 52:997-1005, 1992.

Coukos G, Rubin S. Chemotherapy Resistance in Ovarian Cancer: New molecular perspectives. Gyn Oncol 91:783-790, 1998.

Cramer DW, Welch WR. Determinant of ovarian cancer risk. II Inferences regarding pathogenesis. J Natl Cancer Inst 75:717-721, 1983.

CZernobilsky B, Moll R, Levy R, Franke WW. Coexpression of cytokeratin and vimentin filaments in mesothelial, granulosa, and rete ovarii cells of the human ovary. Eur J Cell Biol 37:175-190, 1985.

Dabholkar M, Bostick-Bruton F, Weber C, Bohr VA, Egwuagu C, Reed E. ERCC1 and ERCC2 expression in malignant tissues from ovarian cancer patients. J Natl Cancer Inst 84:1512-1517, 1992.

Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ, Reed E. Messanger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum chemotherapy. J Clin Invest 94:703-708, 1994.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery. Cell 91:231-241, 1997.

Delclos L, Smith JB. Ovarian Cancer, with special regard to types of radiotherapy. Natl Can Inst Mono 42:129-138, 1975.

Delmastro DA, Li J, Vaisman A, Solle M, Chaney SG. DNA damage inducible-gene expression following platinum treatment in human ovarian carcinoma cell lines. Cancer Chemo Pharm 39: 245-253, 1997.

Deuchars K, Ling VV. P-glycoprotein and multidrug resistance in cancer chemotherapy. Semin Oncol 16:156-165 1989.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388:300-304, 1997.

Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and ctyochrome c by direct inhibition of distinct caspases. EMBO J 17:2215-2223, 1998.

DiSaia PJ, Sinkovics JG, Rutledge FN, Smith JP. Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors. Am J Obs Gyn 114:979-989, 1972.

DiRenzo MF, Olivero M, Katsaros D, Crepaldi T, Gaglia P, Zola P, Sismondi P, Comoglio PM. Overexpression of the MET/HGF receptor in ovarian cancer. Int J Cancer 58:658-662, 1994.

Duckett DR, Drummond JT, Murchie AI, Reardon JT, Sancar A, Lilley DM, Modrich P. Human MutSalpha recognizes damaged DNA base pairs containing o6-methylguanine, o4-methylthymine, or the cisplatin-d(GpG) adduct. Proc Natl Acad Sci USA 93:6443-6447, 1996.

Eastman A. The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. Pharmacol Ther 34:155166, 1987.

Eischen CM, Kottke TJ, Martins LM, Basi GS, Tung JS, Earnshaw WC, Leibson PJ, Kaufmann SH. Comparison of Apoptosis in Wild-Type and Fas-Resistant Cells: Chemotherapy-Induced Apoptosis is not dependent on Fas/Fas Ligand Interactions. Blood 90:935-943, 1997.

El-Deiry WS, Tokinot T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppressor. Cell 75:817-825, 1993.

Eliopoulos AG, Kerr DJ, Herod J, Hodgkins L, Krajewski S, Reed JC, Young LS. The control of apoptosis and drug resistance in ovarian cancer: Influence of p53 and Bcl-2. Oncogene 11:1217-1228, 1995.

Emons G, Ortmann O, Pahwa GS, Hackenberg R, Oberheuser F, Schulz KD. Intracellular actions of Gonadotropic and peptide hormones and the therapeutic value of GnRH-agonists in ovarian cancer. Acta Obst Gyn Scand-Supplement 155:31-38, 1992.

Enari M, Sakahira H, Yokoyama H Okawa K Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43-50,1998.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phospatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immuno 148:2207-2216, 1992.

Fan S, Smith ML, Rivet DJI, Duba D, Zhan KW, Fornace J, O'Conner PM. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res 55:1649-1654, 1995.

Fathalla MF. Incessant ovulation-A factor in ovarian neoplasia? Lancet 2:163,1971.

Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapini JA, Tomaselli KJ, Litwack G, Alnemri ES. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc. Natl. Acad. Sci. U.S.A. 93, 7464-7469, 1996.

Friesen C, Herr I, Krammer PH, Debatin KM. Involvemnet of the CD95(APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nature Med 2:574-77, 1996.

Friesen C, Fulda S, Debatin KM. Deficient activation of the CD95 (APO-1/Fas) system in drug-resistant cells. Leukemia 11:1833-1841, 1997.

Fuchs EJ, McKenna KA, Bedi A. p53-dependent DNA Damage-induced Apoptosis Requires Fas/APO-1-independent Activation of CPP32. Cancer Res 57: 2550-2554, 1997.

Fulda S, Los M, Friesen C, Debatin KM. Chemosensitivity of solid tumor cells in vitro is related to activation of the CD95 system. Int J Cancer 76:105-114, 1998.

Gallion HH, Kohn EC, Mills GB, Bast Jr RC. Clinical Applications of Basic Science Investigations. In Gershenon DM, McGuire WP eds. Ovarian Cancer. New York: Raven Press 357-397, 1998.

Gamen S, Anel A, Lasierra P, Alava MA, Martinez-Lorenzo MJ, Pineiro A Naval J. Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 activation in a Fas-independent way. FEBS Lett 417:360-364, 1997.

Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 119: 493-501, 1992.

Gately DP, Howell SB. Cellular accumulation of the anticancer agent cisplatin: A review. Br J Cancer 67:1171-1175, 1993.

Giordano C. Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Science 275:960-963, 1997.

Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc Natl Acad Sci USA 8:3070-3074, 1992.

Godwin K, Testa JR, Handel LM, Liu Z, Vanderveer L, Tracey P, Hamilton TC. Spontaneous Transformation of Rat Ovarian Surface Epithelium Cells: Association With Cytogenetic Changes and Implications of Repeated Ovulation in the Etiology of Ovarian Cancer. J Nat Can Inst 84:592-601, 1992.

Godwin AK, Vanderveer L, Schultz DC, Lynch HT, Altomare DA, Bultow KH, Daly M Gehsl A, Masny A, Rosenblum N. A common region of deletion on chromoscme 17q in both sporadic and familial epithelial ovarian tumors distal to BRCA1. Am J Hum Genet 55:666-677, 1994.

Goldstein L. MDR1 gene expression in solid tumors. Eur J Cancer 32A:1039-1050, 1996.

Gore-Langton RE, Armstrong DT. Follicular steroidogenesis and its control. In Knobil E, Neill J, ed. The physiology of reproduction. New York:Raven Press, 331-385, 1988.

Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjunent-Brumage H, Tempo P, Korsemeyer SJ. Caspase cleaved BID Targets Mitochondria and is Required for cytochrome c release while BCL-X_L prevents this release but not Tumor necrosis factor-R1/Fas death. J Biol Chem 274:1156-1163, 1999.

Guchelaar HJ, Vermes I, Koopmans RP, Reutelingsperger CPM, Haanen C. Apoptosis- and necrosis-inducing potential of cladribine, cytarabine, cisplatin, and 5fluorouracil in vitro: a quantitative pharmacodynamic model. Cancer Chemother Pharmacol 42:77-83, 1998.

Hahne M, Rimoldi D, Schröter M, Romero P, Schreier M, French LE, Schneider P, Bornanad T, Fontana A, Lienard D, Cerottini JC, Tschopp J. Melanoma cell expression of Fas (APO-1/CD95) ligand:implications for tumor immune escape. Science 274:1363-1366, 1996.

Hale AJ, Smith CA, Sutherland LC, Stoneman EA, Longthorne VL, Culhane AC, Williams GT. Apoptosis: molecular regulation and cell death. Eur J Biochem 236:1-26, 1996

Hamilton TC, Davies P, Griffith K. Oestrogen receptor-like binding in the surface epithelium of the rat ovary. J Endocrinol 95:377-385, 1982.

Hamilton TC,Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McKoy WM, Young RC, Ozols RF. Augmentation of adriamycin, melphalan, and cisplatin toxicity in drug-resistant and sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem Pharmacol 4:2583-2586 1985.

Harder HC, Rosenberg B. Inhibitory effects of anti-tumor platinum compounds on DNA, RNA and protein synthesis in mammalian cells in vitro. Int J Cancer 6:207-216, 1970.

Havrilesky LJ, Elbedary A, Hurteau JA, Whitaker RS, Rodriquez GC, Berchuck A. Chemotherapy-induced apoptosis in epithelial ovarian cancer. Obs Gyn 85:1007-1010, 1995.

Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriquez GC, Bast RC, Berchuck A. Regulation of Apoptosis in Normal and Malignant Ovarian Epithelial Cells by Transforming Growth Factor beta 1. Cancer Res 55:944-948, 1995.

Herod JJO, Eliopoulos AG, Warwick J, Niedobitek G, Young LS, Kerr DJ. The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma. Cancer Res 56:2178-2184, 1996.

Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol 124:43-101, 1991.

Hoffmann JS, Pillaire MJ, Maga G, Podust V, Hubscher U, Villani G. DNA polymerase beta bypasses in vitro a single d(GpG)-cisplatin adduct placed on codon 13 of the HRAS gene. Proc Natl Acad Sci USA 92:5356-5360, 1995.

Holzmayer TA, Hilsenbeck S, Von Hoff DD, Roninson IB. Clinical correlates of MDR1 (P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas. J Natl Cancer Inst 84:146-1491, 1992.

Homburg CHE, de Hass M, von dem Borne AEGK, Verhoeven AJ, Reutelingsperger CPM, Roos D. Human neutrophils lose their surface FcγRIII and acquire Annexin binding sites during apoptosis in vitro. Blood 85:532-540 1995.

Hsueh AJW, Adashi EY, Jones PBC, Welsh TH. Hormonal regulation of the differentiation of cultured granulosa cells. Endocrine Rev 5:76-127, 1984.

Hsueh AJW Billig H, Tsafriri A. Ovarian follicular atresia: a hormonally controlled apoptotic process. Endocrine Rev 15: 707-724, 1994.

Hu S, Vincenz C, Hi J, Gentz R, Dixit VM. I-FLICE, a Novel Inhibitor of Tumor Necrosis Factor Receptor-1 and CD-95-induced Apoptosis. J Biol Chem 272:17255-17257, 1997.

Hu Y, Benedict MA, Wu D, Inohara N, Nunez G. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc Natl Acad Sci USA 95:4386-4391, 1998.

Huang JC, Zamble DB, Reardon JT, Lippard SJ, Sancar A. HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. Proc Natl Acad Sci USA 91:10394-10398, 1994.

Humke EW, Ni , Dixit VM. ERICE a Novel FLICE-activatable Caspase. J Biol Chem 273:15702-15707 1998.

Husain A, He G, Venkatraman ES, Spriggs DR. *BRCA1* Up-Regulation Is Associated with Repair-mediated Resistance to *cis*-Diamminedichloroplatinum (II). Cancer Res 58:1120-1123 1998.

Hsu SC, Wu CC, Luh TY, Chou CK, Han SH, Lai MZ. Apoptotic signal of Fas is not mediated by ceramide. Blood 91:2658-2663, 1998.

Ishikawa T, Ali-Osman F. Glutathione-associated cis-diamminedichloroplatinum (II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. J Biol Chem 268:20116-20125, 1993.

Isola I, Kallionemi OP, Korte JM, Wahistron T, Aine R, Helle M, Helin H. Steroid receptors and Ki-67 reactivity in ovarian cancer and in normal ovary: Correlation with DNA flow cytometry, biochemical receptor assay and patient survival. J Path 162:295-301, 1990.

Isonishi S, Andrews PA, Howell SB. Increased sensitivity to cis-diamminedichloroplatinum (II) in human ovarian carcinoma cells in response to treatment with 12-O-tetracdecanoylphorbal-13-acetate. J Biol Chem 265:3623-3627, 1990.

Isonishi S, Hom DK, Thiebaut FB, Mann SC, Andrews PA, Basu A, Lazo JS, Eastman A, Howell SB. Expression of the c-Ha-ras oncogene in mouse NIH 3T3 cells induces resistance to cisplatin. Cancer Res 51:5903-5909, 1991.

Isonishi S, Jekunen AP, Hom DH, Eastman A, Edelsetein PS, Thiebaut FB, Christen RD, Howell SB. Modulation of cisplatin sensitivity and growth rate of an ovarian carcinoma cell line by bombesin and tumour necrosis factor-α. J Clin Invest 90:1436-1442, 1992.

Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66: 233-243, 1991.

Jacobson MD, Burne JF, Raff MC. Programmed cell death and bcl-2 protection in the absence of a nucleus. EMBO J 13:1899-1910, 1994.

Jacobson MD, Weil M, Raff MC. Programmed cell death in animal developement. Cell 88: 347-354, 1997.

Johnson SW, Perez RP, Godwin AK, Yeung AT, Handel LM, Ozols RF, Hamilton TC. Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. Biochem Pharmacol 47:689-697, 1994.

Johnson SW, Laub PB, Beesley JC, Ozols RF, Hamilton TC. Increased platinum and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. Cancer Res 57:850-856, 1996.

Jürgensmeier JM, Xie Z, Deveraux QL, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci USA 95:4997-5002 1998.

Kacinski BM, carter D, Mittal K, Yee LD, Scata KA, Donofrino L, Chamber SK, Wang KI, Yang-Feng T, Rochrschneider LR. Ovarian adenocarcinomas express fms-complementary transcripts and fms antigen, often with co-expression of CSF1. Am J Pathol 137:135-141, 1990.

Kacinski BM, Stanley ER, Carter D, Chambers JT, Chambers SK, Kohorn EI, Schwartz PE. Circulating levels of CSF-1 (M-CSF) a lymphohematopoietic cytokine may be a useful marker of disease status in patients with malignant ovarian neoplasm. Int J Radiat Oncol Biol Phys 17:159-164, 1989.

Kavallaris M, Leary J, Barrett JA, Friedlander ML. MDR1 and multidrug resistance-associated protein (MRP) gene expression in epithelial ovarian tumors. Cancer Lett 102:7-16, 1996.

Kasibhatla S Brunner T, Genestier L, Echeverri F Mahboubi A, Green DR. DNA Damaging Agents Induce Expression of Fas Ligand and Subsequent Apoptosis in T Lymphocytes via the Activation of NF-κB and AP-1. Mol Cell 1:543-551, 1998.

Keane MM, Ettenberg SA, Lowrey GA, Russell EK, Lipkowitz S. Fas expression and function in normal and malignant breast cancer cell lines. Cancer Res 56:4791-4798, 1996.

Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH, Lazo JS. Overexpression of metallothionein confers resistance to anticancer drugs. Science 241:1813-1815, 1988.

Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenonmenon with wide ranging implications in tissue kinetics. British J Cancer 26: 239-257, 1972.

Kerr JFR, Winterford CM, Harmon BVV. Cell Biology: a laboratory handbook In Celis JE ed. Morphological criteria for identifying apoptosis. San Diego: Academic Press, 319-329, 1994.

Kim JM, Boone DL, Auyeung A, Tsang BKT. Granulosa Cell Apoptosis Induced at the Penultimate Stage of Follicular Development is Associated with Increased Levels of Fas and Fas Ligand in the Rat Ovary. Biol of Repro 58: 1170-1176, 1998.

Kim JM, Yoon YD, Tsang BK. Involvement of the Fas/Fas Ligand System in p53-Mediated Granulosa Cell Apoptosis during Follicular Development and Atresia. Endo 140:2307-2317, 1999.

Knight RL, Hand D, Piacentini M, Griffin M. Characterization of the transglutaminase-mediated large molecular mass polymer from rat liver; its relationship to apoptosis. Eur J Cell Biol 60:210-216, 1993.

Kohler MF, Marks JR, Wiseman RW, Jacobs IJ, Daudoff AM, Clarke-Pearson DL, Soper JT, Bast RC Jr, Berchuck A. Spectrum of mutation and frequency of allelic deletion of the p53 gene in ovarian cancer. J Natl Cancer 85:1513-1519, 1993.

Kondo H, Maruo T, Peng X, Mochizuki M. Immunological Evidence for the Expression of the Fas Antigen in the Infant and Adult Human Ovary during Follicular Regression and Atresia. J Clin Endo Metab 81:2702-2710, 1996.

Kroning R, Jones JA, Hom DK, Chuang CC, Sanga R, Los G, Howell SB, Christen RD. Enhancement of drug sensitivity of human malignancies by epidermal growth factor. Br J Cancer 72:615-619, 1995.

Kruk PA, Auersperg N. Human ovarian surface epithelial cells are capable of physically resttructuring extracellular matrix. Am J Obstetr Gynecol 167:1437-1443 1992.

Kruk PA, Uitto V, Firth JD, Dedhar S, Auersperg N. Reciprocal interactions between human ovarian surface epithelial cells and adjacent extracellular matrix. Exp Cell Res 215:97-108, 1994.

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384:368-372, 1996.

Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.

Lai G-M, Ozols R, Smyth J, Young RC, Hamilton TC. Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. Biochem Pharmacol 37:4597-4600, 1988.

Lai G-M, Ozols R, Young R, Hamilton T. Effect of glutathione on DNA repair in cisplatin resistant human ovarian cancer cell lines. J Natl Cancer Inst 81:535-539, 1989.

Lee S, Elenbaas B, Levine AJ, Griffith J. p53 and its 14kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell 81:1013-1020, 1995.

Lewis A, Hayes J Wolf C. Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: Intrinsic differences and cell cycle effects. Carcinogenesis 9:1283-1287, 1988.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489, 1997.

Lieberthal W, Triaca V, Levine J. Mechansims of death induced by cisplatin in proximal tubular epithelial cell: apoptosis vs. necrosis. Am J Physiol 270:F700-708, 1996.

Lipner H. Mechanism of mammalian ovulation. In Knobil E, Neill J, eds. The physiology of reproduction. New York:Raven Press, 447-488, 1988.

Liu Y, Bhalla K, Hill C, Priest DG. Evidence for Involvement of Tyrosine Phosphorylation in Taxol-Induced Apoptosis in a Human Ovarian Tumor cell line. Biochem Pharm 48:1265-1272, 1994.

Liu X, ou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175-184, 1997

Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NFkB activation prevents cell death. Cell 87:565-576, 1996.

Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74:957-967, 1993.

Louie KG, Behrens BC, Kinsella TJ, Hamilton TC, Grotzinger KR, McKoy WM, Winker MA, Young RC and Ozols RF. Radiation survival parameters of antineoplastic drug-sensitive and –resistant human ovarian cancer cell lines and their modification of buthionine sulfoximine. Cancer Res 45:2110-2115, 1985.

Ma J Maliepaard M, Kolker HJ Vverweij J, Schellens JHM. Abrogatd energy-dependent uptake of cisplatin in a cisplatin-resistant subline of the human ovarian cancer cell line IGRO-1. Cancer Chemother Pharmacol 41:186-192, 1998.

Mamenta EL, Poma EE, Kaufmann WK, Delmastro DA, Grady HL, Chaney SG. Enhanced replicative bypass of platinum-DNA adducts in cisplatin-resistant human ovarian carcinoma cell lines. Cancer Res 54:3500-3505, 1994.

Mann SC, Andrews PA, Howell SB. Modulation of cis-diamminedichloroplatinum (II) accumulation and sensitivity by forskolin and 3-isobutyl-1-methylxanthine in sensitive and resistant human ovarian carcinoma cells. Int J Cancer 48:866-872, 1991.

Marks JR, Davidoff AM, Kerns BJ, Humphrey PA, Pena JC, Dodge RK, Clarke-Pearson DI, Iglehart JD, Bast RC Jr, Berchuck A. Overexpression and mutation of p53 in epithelial ovarian cancer. Cancer Res 51:2979-2984, 1991.

Martin DA, Siegel RM, Zheng L, Lenardo MJ. Membrane Oligomerization and Cleavage activates the caspase-8 (FLICE/MACH1) Death signal. J Biol Chem 273:4345-4349, 1998.

Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson Jr EM. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol 106:829-844, 1988.

Martin SJ, Cotter TG. Disruption of microtubules induces an endogenous suicide pathway in human leukemia HL-60 cells. Cell Tissue Kinet 23:545-559, 1990.

Martinez-Lorenzo MJ, Gamen S Etxeberra P, Lasierra P, Larrad L, Pineiro A, Anel A, Naval J, Alava MA. Resistance to apoptosis correlates with a highly proliferative phenotype and loss of Fas and CPP32 (Caspase-3) expression in human leukemia cells. Int J Cancer 7:473-481, 1998.

Marquis ST, Rajan J, Wynshaw-Boris A, Xu J, Yin GY, Abel KJ, Weber BL, Chodosh LA. The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. Nature Genetics 11:17-26, 1995.

Masuda H, Tanaka T, Matsuda H, Kusaba I. Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to cis-diamminedichloroplatium (II). Cancer Res 50:1863-1866, 1990.

Medema JP, Scaffidi C, Krammer PH, Peter ME. Bcl-xL Acts Downstream of Caspase-8 Activation by the CD95 Death-Inducing Signaling Complex. J Biol Chem 273: 3388-3393, 1998.

Mello JA, Acharya S, Fishel R, Essigmann JM. The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin. Chem Biol 3:579-589, 1996.

McCurrach ME, Connor TM, Knudson CM, Korsmeyer SJ, Lowe SW. Bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proc Natl Acad Sci USA 94:2345-2349, 1997.

Minn AJ, Rudin CM, Boise LH, Thompson CB. Expression of Bcl-XL can confer a multidrug resistance phenotype. Blood 86:1903-1910, 1995.

Mirakhur B, Parekh HK, Simpkins H. Expression of the Cisplatin Resistance Phenotype in a Human Ovarian Carcinoma Cell Line Segregates with Chromosome 11 and 16. Cancer Res 56:2256-2262, 1996.

Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. Blood 1:151-157, 1993.

Morimoto H, Yonehara S, Bonavida B. Overcoming tumor necrosis factor and drug resistance of human tumor cell lines by combination treatment with anti-Fas antibody and drugs or toxins. Cancer Res 53: 2591-2596, 1993.

Moscatello, DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zottrick PW, Biegel JA, Hayes RL, Wong AJ. Frequent expression of a mutant EGF receptor in multiple human tumors. Cancer Res 55:5536-5539,1995.

Müller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofmann HJ, Stremmel W, Krammer PH, Galle PR. Drug-induced Apoptosis in Hepatoma Cells is Mediated by the CD95 (APO-1/Fas) Receptor/Ligand System and Involves Activation of Wild-type p53. J Clin Inv 99: 403-413, 1997.

Murdoch WJ. Programmed cell death in preovulatory ovine follicles. Biol of Repro 53: 8-12, 1995.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rouke K, Shevchenko A Ni J Scaffidi C Bretz JD, Zhang M, Gentz R. FLICE a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signalling complex. Cell 85:817-827, 1996.

Naik P, Karrim J, Hanahann D. The rise and fall of apoptosis during mutistage tumorigenesis: down-modulation contibutes to tumor progression from angiogenic progenitors Genes & Development 10:2105-2116, 1996.

Nagata S, Golstein P. The Fas Death Factor. Science 267:1449-1455, 1995.

Nagata S. Fas ligand and immune evasion. Nature Med 2:1306-1307, 1996.

Nagata S. Apoptosis by death factor. Cell 88:355-365 1997.

Nambu Y, Hughes SJ, Rehemtulla A, Hamstra D, Orringer MB, Beer DG. Lack of Cell Suface Fas/APO-1 Expression in Pulmonary Adenocarcinomas. J Clin Invest 101:1102-1110, 1998.

Nicholson DW, Thornberry NA. Caspases:killer proteases. TIBS 22:299-306, 1997.

Nicosia SV. Morphological changes in the human ovary throughout life. In G.B. Serra ed. The Ovary. New York:Raven Press, 57-81, 1983.

Nicosia SV, Nicosia R. Neoplasms of the ovarian mesothelium. In Azar H ed. Pathology of Human Neoplasms. New York: Raven Press, 435-486, 1988.

O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack:Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 184:1075-1082, 1996.

O'Dwyer P, Hamilton T, Young RL, LaCreta FP, Carp N, Tew KD, Padavick K, Comis RL, Ozols RF. Depletion of glutathione in normal and malignant human cells in vivo by buthionine sulfoximine: Clinical and biochemical results. J Natl Cancer Inst 84:264-267, 1992.

Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S. Lethal effect of the anti-Fas antibody in mice. Nature 364:806-809, 1993.

Ohmori T, Arteaga CL. Protein Kinase C epsilon translocation and phosphorylation by cis-diamminedichloroplatinum (II) (CDDP): Potential role in CDDP-mediated cytotoxicity. Cell Death Diff 99: 345-353, 1998.

Orlinick JR, Elkon KB, Chao MV. Separate Domains of the Human Fas Ligand Dictate Self-association and Receptor Binding. J Biol Chem 272:32221-32229 1997.

Ormerod MG, O'Neill CF, Robertson D, Harrap KR. Cisplatin Induces Apoptosis in a Human Ovarian Carcinoma cell line without Concomitant Internucleosomal Degradation of DNA. Exp Cell Res 211: 231237, 1994.

Ormerod MG, O'Neill C, Robertson D, Kelland LR, Harrap KR. *cis*-Diamminedichloroplatinum (II)-induced cell death through apoptosis in sensitive and resistant human ovarian carcinoma cell lines. Cancer Chemo Pharm 37: 463-471, 1996.

Ouchi T, Monteiro ANA, August A, Aaronson SA. BRCA1 regulates p53-dependent gene expression. Proc Natl Acad Sci USA 95:2302-2306, 1998.

Owen-Schaub LB, Radinsky R, KrunzelE, Berry K, Yonehara S. Anti-Fas on on hematopoietic tumors:levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. Cancer Res 54:1580-1586, 1994.

Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santes M, Fujiwara T, Roth JA, Deisseroth AB, Kruzel E. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol 15: 3032-3040, 1995.

Ozols RF, Rubin SC Thomas G, Robboy S. Priniciples and practice of gynecologic oncology In Hoskins WJ, Perez CA, Young RC eds. Epithelial ovarian cancer. Philadelphia: Lippincott-Raven, 919-987, 1997.

Pan G, O'Rouke K, Dixit M. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J Biol Chem 273:5841-5845, 1998.

Parekh H, Simpkins H. Species-specific differences in taxol transport and to cytotoxicity against human and rodent cells. Biochem Pharmacol 51:301-311, 1996.

Parker RJ, Eastman A, Bostick-Bruton F, Reed E. Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. J Clin Invest 87:772-777, 1991.

Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, Pierotti MA, Miyashita T, Reed JC, Zunino F. Association between Cisplatin Resistance and Mutation of p53 Gene and Reduced Bax Expression in Ovarian Carcinoma Cell Systems. Cancer Res 56: 556-562, 1996.

Perez C, Albert I, DeFay K, Zachariades N, Gooding L, Kriegler M. A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell to cell contact. Cell 63:251-258, 1990.

Perez CA, Hall EJ, Purdy JA, Williamson J Principles and Practice of Gynecologic Oncology. In Hoskino WJ, Perez CA, Young RC. eds. Biologic and Physical Aspects of Radiation Oncology. Philadelphia: Lippincott-Raven, 305-380, 1997.

Peter MC, Kischkel FC Hellbardt S, Chinnaiyan AM, Krammer PH, Dixit VM.CD95(APO-1/Fas)-associating signalling proteins. Cell Death Diff 3:161-170, 1996.

Piacentini M, Davies PJA, Fesus L. Tissue transglutaminase in cells undergoing apoptosis In Tomei D, Cope FO ed. Apoptosis II: The molecular basis of apoptosis in disease.. New York :Cold Spring Harbor Laboratory Press,143-163, 1994.

Quirk SM, Cowan RG, Joshi SG, Henrikson KP. Fas Antigen-Mediated Apoptosis in Human Granulosa/Luteal Cells. Biol Rep 52:279-287, 1995.

Radisavljevic S. The pathogenesis of ovarian inclusion cysts and cystomas. Obstetr Gynecol 49:424-429 1976.

Raff MC. Social controls on cell survival and cell death. Nature 356:397-400, 1992.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizsaki Y. Programmed cell death and the control of cell survival: lessons from the nervous system. Science 262:695-700, 1993.

Reed JC. Cytochrome c: Can't Live with it-Can't Live without it. Cell 91:559-562, 1997.

Reed JC, Toshiyuki M, Takayama S, Wang H-G, Sato T, Krajewski S, Aime-Sempe C, Bodrug S, Kitada S, Hanada M. Bcl-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 60: 23-32, 1996.

Rensing-Ehl A, Frei K, Flury R, Matiba B, Mariani SM, Weller M, Aebischer P, Krammer PH, Fontana A. Local Fas/APO-1 (CD95) ligand-mediated tumour cell killing in vivo. Eur J Immun 25: 2253-2258, 1995.

Righetti SC, Della Torre G, Pilotti S, Menard D, Ottone F, Colnaghi MI, Pierotti MA, Lavarino C, Cornarotti M, Oriana S, Bohm S, Bresciani GL, Spatti G, Zunino F. A comparative study of p53 gene mutations, protein accumulation, and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. Cancer Res 56:689-693, 1996.

Risma H, Hirshfield AN, Nilson JH. Elevated luteinizing hormone in prepubertal transgenic mice causes hyperandrogenism, precocious puberty, and substantial ovarian pathology. Endo 138:3540-3547, 1997.

Robboy SJ, Duggan M, Kurman RT. The female reproductive system. In: Rubin E, Farber J, eds. Pathology, 2nd Ed. Philadelphia: JB Lippincott, 1988.

Rosenberg B, Van Camp L,Grimely ED, Thomson AJ. The inhibition of growth or cell division in Escherichia coli by different ionic species of platinum (IV) compounds. J Biol Chem 242: 1347-1352, 1967.

Rosl F. A Simple and Rapid Method for Detection of Apoptosis in Human Cells. Nuc Acids Res 20: 5243-5250, 1992.

Ross GT, Schreider JR. Reproductive Endocrinology In Yen SSC, Jaffe RB eds. The Ovary. Philadelphia: W.B. Saunders Company, 115-139, 1986.

Rouquet N, Allemand I, Molina T, Bemour M, Briand P, Joulin V. Fas-dependent apoptosis is impaired by SV40 T-antigen in transgenic liver. Oncogene 11;1061-1067, 1995.

Saburi Y, Nakagawa M, Ono M, Sakai M, Muramatsu M, Kohnok K, Kuwano M. Increased expression of glutathione S-transferase gene in cis-diamminedichloroplatinum (II)-resistant variants of a Chinese hamster ovary cell line. Cancer Res 4:7020-7025 1989.

Sakamaki K, Yoshida H, Nishimura Y, Nishikawa SI, Manabe N, Yonehara S. Involvment of fas antigen in follicular atresia and luteolyisis. Mol Reprod Dev 47:11-18, 1997.

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: a laboratory manual. In Nolan C ed. 2nd edition Cold Spring Harbour Laboratory Press New York, 6.6-6.8, 6.12-6.14, 6.20, 1989.

Savill J. The inate immune system: recognition of apoptotic cells. In Gregory CD ed. Apoptosis and the Immune Response. New York :Wiley-Liss, 341-369, 1995.

Scaffidi C, Medema JP, Krammer PH, Peter ME. FLICE is Predominantly Expressed as Two Functionally Active Isoforms, Caspase-8/a and Caspase-8b. J Biol Chem 272:26953-26958, 1997.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin K-M, Krammer PH, Peter ME Two CD95 (APO-1/Fas) signaling pathways. EMBO J 17:1675-1687, 1998.

Schilder RJ, Hall L, Monks A, Handel LM, Fornace J, Ozols RF, Fojo AT. Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. Int J Cancer 45:416-422, 1990.

Schlegel T, Peter I, Orrenius S, Miller OK, Thornberry NA, Yamin TT, Nicholson DW. CPP32/Apopain is a key Interleukin 1β Converting Enzyme like Protease Involved in Fas mediated Apoptosis. J Biol Chem 271:1841-1844, 1996.

Schwartz LM, Osborne BA. Programmed cell death, apoptosis and killer genes. Immunol Today 14:582-590, 1993.

Scully R, Chen J, Plug A, Xiao Y, Weaver D Feunteun J, Ashley T, Livingston DM. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell 88: 265-75, 1997.

Siemens CH, Auersperg N. Serial propagation of human ovarian surface epithelium in tissue culture. J Cell Physiol 134:347-536, 1988.

Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB, Gray JW. *PIK3CA* is implicated as an oncogene in ovarian cancer. Nat Gent 21: 99-102, 1999.

Sheikh MS, Burns TF, Huang Y, Wu SG, Amundson S, Brooks KS, Fornace AJ, El-Deiry WS. P53-dependent and –independent regulation of the Death Receptor Killer/DR5 Gene expression in Response to Gentoxic Stress and Tumor Necrosis Factor α. Cancer Res 58:1593-1598, 1998.

Sherman SE, Lippard SJ. Structural aspects of platinum anticancer drug interaction with DNA. Chem Rev 87: 1153-1157, 1987

Skilling JS, Squatrito RC, Connor JP, Niemann T, Buller RE. p53 gene mutation analysis and antisense-mediated growth inhibition of human ovarian carcinoma cell lines. Gyn Oncol 60: 72-80, 1996.

Smith A, Ramos-Morales F, Ashworth A, Collins M. A role for JNK/SAPK in proliferation, but not apoptosis, of IL-3-dependent cells. Curr Biol 7:893-896, 1997

Soldatenkov VA, Prasad S, Notario V, Dritschilo A. Radiation-induced apoptosis of Ewings's sarcoma cells: DNA fragmentation and proteolysis of poly (ADP-ribose) polymerase. Cancer Res 55: 4240-4242, 1995.

Song K, Li Z, Seth P, Cowan KH, Sinha BK. Sensitization of cis-platinum by a recombinant adenovirus vector expressing wild-type p53 gene in human ovarian carcinomas. Oncol Res 9:603-609, 1997.

Sorengo MS, Alarcon RM, Yoshide H, Giaccia AJ, Hakem R, Mak TW, Lowe SW. Apaf-1 and Caspase-9 in p53-dependent apoptosis and tumor inhibition. Science 284:156-159, 1999.

Sorenson CM, Barry MA, Eastman AJ. Analysis of events associated with cell cycle arrest at G2 phase and cell and cell death induced by cisplatin. J Natl Cancer Inst 82: 749-755, 1990.

Soussi T, Caron de Fromentel C, May P. Structural aspects of the p53 protein relation to gene evolution. Oncogene 5:945-952, 1990.

Stehlik C, de Martin R, Kumbashiri I Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-kappaB regulated X-chromosome linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J Exp Med 188:211-216 1998.

Stenwig JT, Hazekamp JT, Beecham JB. Granulosa cell tumours of the ovary: A cliniopathological study of 118 cases with long-term follow up. Gynecol Oncol 7:136-152, 1979.

Stewart DJ, Raaphorst GP, Yau J, Beaubien AR. Active vs. passive resistance, dose-response relationships, high dose chemotherapy, and resistance modulation: a hypothesis. Invest New Drugs 14: 115-130, 1996.

Suda T, Nagata S. Purification and characterization of the Fas-ligand that induces apoptosis. J Exp Med 179: 873-879, 1994.

Sun XM, MacFarlane M, Zhuang J Wolfs BB, Green DR, Cohen GM. Distinct Caspase Cascades are initiated in Receptor-mediated and Chemical-induced Apoptosis. J Biol Chem 274:5053-5060, 1999.

Takahashi T, Tanaka M, Inazawa J, Abe T, Suda T, Nagata S. Human Fas Ligand:gene structure, chromosomal location and species specificity. Int Immun 6:1567-1574, 1994.

Takahashi H, Behbakht K, McGovern PE, Chiu H, Couch FJ, Weber BI, Friedman LS, King M, Furusato M, Livolsi VA, Menzin AW, Liu PC, Benjamin I, Morgan MA, King SA, Rebane BA, Cardonick A, Mikuta JJ, Rubin SC, Boyd J. Mutation analysis of the *BRCA1* gene in ovarian cancers. Cancer Res 55:2998-3002, 1995.

Tamiya S, Etoh K, Suzushima H, Takatsuki K, Matsuoka M. Mutation of CD95 (Fas/APO-1) Gene in Adult T-cell leukemia. Blood 91;3935-3942, 1998.

Tanaka M, Itai T, Adachi M, Nagata S Downregulation of Fas ligand by shedding. Nature Medicine 4: 31-36, 1998.

Tanaka M, Suda T, Haze K, Nakamura N, Sato K, Kimura F, Motoyoshi K, Mizuki M, Tagawa S, Ohga S, Hatake K, Drummond AH, Nagata S. Fas Ligand in human serum. Nature Medicine 2: 317-322, 1996.

Tanaka M, Suda T, Yatomi T, Nakamura N, Nagata S. Lethal effect of recombinant human Fas ligand in mice pretreated with *Propionibacterium acnes*. J Immunol 158:2303-2309, 1997.

Tanaka M, Suda T, Takahashi T, Nagata S. Expression of the functional soluble from of human Fas Ligand in activated lymphocytes. EMBO J 14: 1129-1135, 1995.

Terasima T, Tolmach LJ. Variations in several responses of Hela cells to x-irradiation during division cycle. Biophys J 3:11-16, 1963.

Thornberry NA, Lazebnik Y. Caspases: Enemies Within. Science 281:1312-1316, 1998.

Tolomeo M, Dusonchet L, Meli M, Grimaudo S, D'Alessandro N, Papoff G, Ruberti G, Rausoa L. The CD95/CD95 ligand system is not the major effector in anticancer drug-mediated apoptosis. Cell Death Diff 5:735-742, 1998.

Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456-1462, 1995.

Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk N, Debatin KM, Krammer PH. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245:301-305, 1989.

Tsang BK, Sheng YL, Sasaki H, Li J, Schneiderman D, Kim JM, Liston P, Fung kee Fung M, Faught W, Senterman M, Korneluk R, Kotsuji F. X-linked Inhibitor of Apoptosis Protien (XIAP) in Human Ovarian Cancer: Role in Chemoresistance and a Possible Target for Gene Therapy. Ovarian Cancer Forum, Toronto, Canada, 1999.

Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 41:1967-1972 1981.

Uslu R, Jewett A, Bonavida B. Sensitzation of Human Ovarian Tumor Cells by Subtoxic CDDP to Anti-Fas Antibody-Mediated Cytotoxicity and Apoptosis. Gyn Oncol 62: 282-291, 1996.

van Dam PA, Lowe DG, Watson JV, James M, Chard T, Hudson CN, Shepherd JH. Multiparameter flow-cytometric quantiation of epidermal growth factor receptor and the c-erbB-2 oncoprotein in normal and neoplastic tissues of the female genital tract. Gynecol Oncol 42:256-264, 1991.

Vasey PA, Jones NA, Jenkins S, Dive C, Brown R. Cisplatin, camptothecin, and taxol sensitivities of cells with p53-associated mutlidrug resistance. Mol Pharmacol 50:1536-1540, 1996.

Vincenz C, Dixit VM. Fas-associated Death Domain Protein Interleukin-1β-converting Enzyme 2 (FLICE2), an ICE/CED-3 Homologue, Is Proximally Involved in CD-95- and p55-mediated Death Signalling. J Biol Chem 272:6578-6583, 1997.

Villunger A, Egle A, Kos M, Hartmann BL, Geley S, Kofler R, Greil R. Drug-induced Apoptosis is Associated with Enhanced Fas (Ap0-1/CD95) Ligand Expression but Occurs Independently of Fas (Apo-1/CD95) Signaling in Human T-Acute Lymphatic Leukemia Cells. Cancer Res 57:3331-3334, 1997.

Wakahara Y, Nawa A, Okamoto T, Hayakawa A, Kikkawa F, Suganuma N, Wakahara F, Tomoda Y. Combination Effect of Anti-Fas Antibody and Chemotherapeutic Drugs in Ovarian Cancer Cells in vitro. Oncol 54: 48-54, 1997.

Wang HG, Rapp UR, Reed JC. Bcl-2 targets the protein kinase raf-1 to mitochondria. Cell 87:629-638, 1996.

Wang XW, Yeh H, Schaeffer L, Roy R, Moncollin VV, Egly JM, Wang Z Friedberg EC, Evans MK. Taffe BG. p53 modulation of TFIIH associated nucleotide excsision repair activity. Nature Genetics 10:188-195, 1995.

Weller M, Malipiero U, Aguzzi A, Reed JC, Fonatana A. Protooncogene bcl-2 Gene Transfer Abrogates Fas/APO-1 Antibody-mediated Apoptosis of Human Malignant Glioma Cells and Confers Resistance to Chemotherapeutic Drugs and Therapeutic Irradiation. J Clin Invest 95:2633-2643 1995.

Williams S, Wong LC, Ngan HYS. Ovarian Cancer In Gershenson DM, McGuire WP Ed. Management of Ovarian Germ Cell Tumours. New York :Churchill Livingstone, 399-415, 1998.

Wu S, Boyer CM, Whitaker RS, Berchuck A, Wiener JR, Weinberg JB, Bast RC. Tumor necrosis factor alpha as an autocrine and paracrine growth factor for ovarian cancer: Monokine induction of tumor cell proliferation and tumor necrosis factor alpha expression. Cancer Res 53:1939-1944, 1993.

Wyllie AH, Kerr FR, Currie AR. Cell death: the significance of apoptosis. Inter Re Cytology 68: 251-306, 1980.

Wyllie AH, Morris RG, Smith AL Dunlop D. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:66-77 1984.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326-1331, 1995.

Xu CF, Solomon E. Mutations of the BRCA1 gene in human cancer. Cancer Biol 7:33-40, 1996.

Yaginuma Y, Yamashita K, Kuzumaki N, Fujita M, Shimizu T. ras oncogene product p21 expression and prognosis of human ovarian tumors. Gynecol Oncol 46:45-50, 1992.

Yang X, Khosravi-Far R, Chang HY, Baltimore D. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell 89: 1067-1076, 1997.

Yao KS, Godwin AK, Johnson SW, Ozols RF, O'Dwyer PJ, Hamiltion TC. Evidence for altered regulation of gamma-glutamylcysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. Cancer Res 55:4367-4374, 1995.

Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mithcell K, Deiry WSE, Lowe SW, Goeddel DV, Mak TW. FADD: Essential for Embryo Development and Signaling from Some, But not All, Inducers of Apoptosis. Science 279:1954-1958, 1998.

Yonehara S, Ishii M, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 169:1747-1756,1989.

Yonehara S. Involvment of apoptosis antigen Fas in clonal deletion of human thymocytes. Int Imm 6:1849-1856, 1994.

Young RC. Principles and Practice of Gynecologic Oncology. In Hoskino WJ, Perez CA, Young RC, Williamson J eds. Principles of Chemotherapy in Gynecologic Cancer. Philadelphia: Lippincott-Raven, 399-456, 1997.

Zhan Q, Carrier F, Fornace AJ. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol Cell Biol 13:4242-4250, 1993.

Zheng W, Magid MS, Kramer EE, Chen YT. Follicle-stimulating hormone receptor is expressed in human ovarian surface epithelium and Fallopian tube. Am J Pathol 148:47-53, 1996.

Zinkewich-Peotti A, Andrews PA. Loss of cis-diamminedichloroplatinum (II) resistance in human ovarian carcinoma cells selected for Rhodamine 123 resistance. Cancer Res 52:1902-1906, 1992.

Ziltener HJ, Maines-Bandiera S, Schrader JW, Auersperg N. Secretion of bioactive IL-1, IL-6 and colony stimulating factors by human ovarian surface epithelium. Biol Reprod 49:635-641, 1993.

Zou H, Henzel WJ, Liu X, Luschg A, Wang X. Apaf-1, a Human Protein Homologous to C.elegans CED-4, Participates in Cytochrome c-Dependent Activation of Caspase-3. Cell 90:405-413, 1997.

17.0 BIBLIOGRAPHY

Name: Danielle Schneiderman

Education

M. Sc.: Physiology, University of Ottawa (1996-1999)

B. Sc. Honours: Biology, University of Waterloo (1990-1995)

Awards and Scholarships

National Awards

1. National Sciences and Engineering Research Council of Canada (NSERC) Post Graduate Scholarship A (1996-1998)

2. The Karen Lackey Summer Studentship award (1994)

Local Awards

3. The Judith E. Raymond Scholarship for Cancer Research (1998)

4. University of Ottawa Excellence Scholarship (1996)

5. University of Waterloo, Faculty of Science Dean's Honor List (1994-1995)

Publications

Schneiderman D, Kim JM, Sentermen M, Tsang BK. Sustained suppression of Fas ligand expression: An important determinant in cisplatin resistance in human ovarian epithelial cancer. Apoptosis 4:271-282, 1999.

Sasaki H, Li J, **Schneiderman D**, Sheng Y, Kim JM, Feng Q, Kotsuji F, Tsang BK. Life, Death and Immortality: Cellular and Molecular Perspectives in Human Ovarian Cancer Growth. Proceedings for IV Sapporo International Symposium on Ovarian Cancer, 1999 (in press).

Henderson JE, Amizuka N, Warshawsky H, **Biasotto D**, Lanske BMK, Goltzman D, Karaplis A. Nucleolar Localization of Parathyroid Hormone-Related Peptide Enhances Survival of

Chondrocytes under conditions that Promote Apoptotic Cell Death. Mol Cell Biol 15:4064-4075, 1995.

Manuscripts in Preparation or Submitted

Schneiderman D, Xiao C, Tsang BK. Cisplatin induced caspase-3 and –8 cleavage and apoptosis in a sensitive but not resistant human ovarian epithelial cancer cells (in progress).

Li J, Feng Q, Kim JM, **Schneiderman D,** Liston P, Li M, Vanderhyden B, Faught W, Fung MFK, Senterman M, Korneluk RG, Tsang BK. Human Ovarian Cancer and Cisplatin Resistance: Possible Role of Inhibitor of Apoptosis Proteins (submitted).

Research Presentations

Schneiderman D, Kim JM, Sentermen M, Tsang BK. Involvement of Fas/FasL system and Apoptosis in Chemoresistance in Human Ovarian Epithelial Cancer. Biol Reprod 56:291 (supplement 1), 1997, presented at the Society for the Study of Reproduction, Portland, Oregeon, USA.

Schneiderman D, Kim JM, Sentermen M, Tsang BK. Ottawa Reproductive Biology Workshop, Ottawa, Ontario, 1997 and 1998.

Sasaki H, Li J, Sheng YL, Feng Q, **Schneiderman D**, Kim JM, Liston P, Korneluk R, Kotsuji F and Tsang BK. Chemoresistance in Human Ovarian Cancer: Possible Role of Inhibitor of Apoptosis Proteins. The 4th Sapporo International Symposium on Ovarian Function, Sapporo, Japan, August 7-8, 1998.

Schneiderman D, Xiao C, Tsang BK. Cisplatin induced caspase-3 and -8 cleavage and apoptosis in a sensitive but not resistant human ovarian epithelial cancer cell. Ottawa Reproductive Biology Workshop, Ottawa, 1999.

Tsang BK, Sheng YL, Sasaki H, Li JL, **Schneiderman D**, Kim JM, Liston P, Fung kee Fung M, Faught W, Senterman M, Korneluk R, Kotsuji F. X-linked inhibitor of apoptosis protein in human ovarian cancer: chemoresistance and a possible target for gene therapy. 55th Anunual Meeting of the Society of Obstetricians and Gynaecologists of Canada, Ottawa, Canada, June 25-29, 1999.