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Plasma Membrane-Derived Shed Vesicles Bearing Growth Regulatory Molecules Induce Cell Proliferation or Cell Death: A Potential Mechanism For Intra/Intercellular Communication

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICINE - DIVISION OF EXPERIMENTAL MEDICINE

McGILL UNIVERSITY

MONTREAL, QUEBEC, CANADA

Abstract

Apoptosis, a mechanism for modulating tissue growth, is triggered by signals (Fas ligand (FasL), tumor necrosis factor-α (TNF-α)) derived from other cells as well as external factors such as ultraviolet radiation (UV). Lipids and proteins associated with the plasma membrane are continually lost from the cell surface on shed vesicles via exfoliation, a directed, energy dependent process that is driven by first order kinetics by physical and biochemical properties of the lipid bilayer. We have investigated the possibility that FasL, Fas, M-CSF and flt3/flk2 ligand are released on shed vesicles from HuT 78 cells, tumor cell lines (MIP-101 and CX-1), and Chinese hamster ovary (CHO) cells transfected with cDNA encoding for M-CSF and flt3/flk2 ligand, respectively. Further, we examined the effects of UV-B irradiation on the capacity of CHO cells to shed vesicles *per se*, and on the capacity of CHO cells transfected with cDNA encoding flt3/flk2 ligand to express flt3/flk2 ligand on their cell surface. CHO cells irradiated with UV-B demonstrated a dose-dependant reduction in viability, as was evidenced by their inability to exclude trypan blue dye, and DNA fragmentation patterns as well as, morphological changes characteristic for apoptosis after 18 hr when exposed to doses of 300 - 2800 Joules/m^2. The release of extracellular shed vesicles was examined in ^{125}I labeled unirradiated (control) and irradiated (600 Joules/m^2) CHO cells. Shed vesicles were collected from serum-free conditioned medium by isopycnic centrifugation at 0 - 18 hr and quantified by scintillation spectrometry. The rate of release was decreased by 57% after 2 hr and cumulative shedding was decreased to 21% after 18 hr. The most dramatic change was observed in the first 4 hr, when no morphological alterations associated with apoptosis were visible. Vesicles and plasma membranes from CHO cells solubilized in N-octyl-β-D-glycopyranoside, electrophoresed in polyacrylamide and reacted

with anti-human M-CSF or anti-flt3/flk2 ligand antibody revealed bands of 90 and 45 kDal or 32 kDal bands, respectively. Following irradiation with UV-B (600 Joules/m^2), CHO cells transfected with flt3/flk2 ligand cDNA failed to express the protein at their cell surface. When plasma membranes and shed vesicles collected from HuT 78 cells, solubilized, electrophoresed in polyacrylamide and reacted with anti-FasL IgG, immunoreactive FasL protein was detected in membranes and vesicles from HuT 78 cells. Equilibrium dialysis and Scatchard plot analysis indicated the presence of 3 to 4 receptor molecules on SVs from MIP-101 cells. That FasL and Fas antigen are present on SVs in a bioactive configuration is revealed by the MTT viability assay. When exposed to FasL-bearing SVs 67.2 ± 0.59% of Fas-expressing tumor cells survived as compared to cells treated with SVs derived from CX-1 cells (control), results which were comparable to the level of cell death observed following treatment with anti-Fas antibody (69.1 ± 1.25%). Tumor cells treated with FasL- and Fas-bearing SVs simultaneously, exhibited 96.4 ± 1.04% viability, indicating that FasL and Fas interact when expressed on SVs, in vitro. Furthermore, Fas antigen-bearing SVs from MIP-101 cells protect tumor cells from anti-Fas-mediated apoptosis. Our results suggest that diminished release of shed vesicles represents a potential early event in radiation-induced apoptosis occurring even prior to plasma membrane blebbing, and adds to the accumulating body of evidence that the signal in radiation-induced apoptosis is propagated from the cell surface to the nucleus. In addition, we provide evidence to suggest that both, ligand and Fas receptor are shed on extracellular vesicles that are derived from the plasma membrane. Since they retain a bioactive configuration, shed vesicles expressing FasL or Fas antigen may interact with the cell of origin or neighboring cells to trigger autocrine and paracrine apoptosis, respectively.

Résumé

L'apoptose, un mécanisme par lequel l'accroissement des tissus est modulé, est déclenchée par des signaux (ligand Fas (FasL), le facteur nécrosant des tumeurs-α (TNF-α)) secrété par d'autres cellules ainsi que, par des facteurs externes, tels que, la radiation ultraviolette (UV). Des Lipides et des protéines associés à la membrane plasmique (PM) sont continuellement relâchés de la surface cellulaire à la surface des vésicules (SVs) en conséquence de l'exfoliation, un processus requérant de l'énergie, et qui est guidé par la cinétique de premier ordre et selon les propriétés physiques et biochimiques de la membrane cellulaire. Nous avons mené des recherches pour déterminer si le FasL, Fas, M-CSF et le ligand flt3/flk2 sont relâchés sur des SVs libérés par des cellules HuT 78, des cellules cancéreuses (MIP-101 et CX-1) et des cellules ovariennes du hamster chinois (CHO) transfectées, respectivement, avec de l'ADN codant pour le M-CSF ou pour le ligand flt3/flk2. De plus, nous avons examiné les influences de l'UV-B sur la capacité des cellules CHO à relâcher des SVs, ainsi que, la capacité des cellules CHO transfectées avec de l'ADN codant pour le ligand flt3/flk2 d'exprimer ce ligand à leurs surfaces. Les cellules exposées au rayonnement UV-B ont démontré une réduction de viabilité proportionnelle à la dose de radiation 18 heures après une exposition de 300 à 2800 Joules/m^2. Ces cellules irradiées sont alors incapables d'exclure le bleu trypan, leurs ADN se fragmente et ils acquièrent des caractères morphologiques attribués à l'apoptose. Nous avons examiné l'exfoliation des SVs provenant de la surface de cellules CHO marquées avec l'isotope ^{125}I mais non-exposées au rayonnement UV-B (contrôle) et des cellules marquées et irradiées par une dose de 600 Joules/m^2. Après 0 à 18 heures, les SVs ont été recueillies du milieu de culture conditionné par des cellules CHO par ultracentrifugation et quantifiées par la suite, avec un compteur à scintillation. La vélocité de l'exfoliation a diminué de 57% après 2 heures et l'exfoliation cumulative à diminué jusqu'à 27% après 18 heures. Le changement le plus dramatique est

survenu après 4 heures, lorsque aucune altération morphologique attribuée à l'apoptose fut remarquée. SVs et PMs dissoutes dans du N-octyl-β-D-glycopyranoside, soumises à l'électrophorèse et analysées avec des anticorps anti-M-CSF ou anti-flt2/flk3 ont révélés respectivement, des bandes de 90 et 45 kDal ou 32 kDal. Un traitement d'UV-B (600 Joules/m^2) a supprimé l'expression du ligand flt3/flk2 à la surface des cellules CHO. Lorsque les PMs et les SVs obtenues de cellules HuT 78 ont été dissoutes, séparées par électrophorèse et sondés avec des anticorps IgG anti-FasL, la protéine FasL, a été détecté dans les PMs et les SVs. L'analyse de Scartchard des données obtenues par dialyse d' équilibre a révélé la présence de 3 à 4 récepteurs Fas à la surface des SVs recueillis des cellules MIP-101. L' étude de viabilité, MTT, a révélé que le FasL et son récepteur, Fas, se retrouvent sur des SVs dans une configuration bioactive. Lorsque cultivées en présence de SVs qui contiennent le FasL (SVs-FasL), 67.2 ± 0.6% des cellules CX-1 ont survécus, comparativement aux cellules cultivées en présence de SVs provenant des cellules CX-1 (contrôle). Ce niveau de viabilité est comparable à celui qu' on observe lorsque ces cellules sont traitées avec des anticorps anti-Fas (69.1 ± 1.2%). Les cellules cancéreuses traitées, simultanément, avec des SVs-FasL ainsi que des SVs-Fas ont démontré un niveau de viabilité de 96.4 ± 1.0%; ceci suggère que, *in vitro*, le FasL et le récepteur Fas se reconnaissent lorsqu' ils se trouvent à la surface des SVs. De plus, SVs-Fas ont pu protéger les cellules CX-1 lorsque ces dernières ont été cultivées en présence des anticorps anti-Fas. Nos résultats suggèrent qu' une diminution de l'exfoliation représente un événement qui apparaît très tôt dans le processus de l'apoptose causé par la radiation UV-B et s'ajoutent aux preuves selon lesquelles ce phénomène prend origine à la surface de la cellule et se répand vers le noyau. En plus, nos recherches suggèrent que le FasL et son récepteur sont libérés sur des SVs. Comme ils retiennent une configuration bioactive, ces SVs peuvent interagir soit avec les cellules d'origine ou d'autres cellules voisines et, ainsi, moduler l'apoptose de ces cellules.

ACKNOWLEDGMENTS

I would like to extend my gratitude to my wife, Suzanne Schreyer, for encouraging me to enter the PhD program and for her continued encouragement throughout the course of my studies.

I wish to thank my parents, Elizabeth and Pasquale Albanese, for without their support and unwavering patience, I could not be involved in the field of Applied Sciences.

I wish to thank research assistants, Sandra Kreczko, Helen Dicso, Maria Kontogiannea, and Kathy Ceccerelli, as well as my fellow graduate students An Jing, Georgi Shablovsky and Martina Timm, for their help with the many techniques described in this document. Thank you Catherine Dubreuil for the enthusiasm you brought to the lab. In addition, I thank Lia Caporicci, and Brigitte Morin of the Cell Marker Laboratory at the Royal Victoria Hospital, and Ken McDonald, of Dr Michael Ratcliffe's laboratory, for flow cytometric analysis of my many, many samples. I am thankful to Dr S. Meterissian for providing cell lines used for experiments presented in this document.

I wish to thank members of my committee, Drs AK. Sullivan, H. Bennett, J. Silvius, and M. Ratcliffe for their input and assistance for the work presented in this thesis. Finally, but certainly not the least of all, my supervisor, Dr Nicholas Dainiak, deserves the highest praise for his persistent and unwavering support during the course of my graduate studies at McGill University and for his critical appraisal of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium
ACID	Activation-induced cell death
AMP	Adenosine monophosphate
BCA	Bicinchoninic acid
BFU-E	Brust-forming unit-erythroid
BGP	Biliary glycoprotein
CAM	Cell adhesion molecule
CHO	Chinese hamster overy
cDNA	Copy deoxynucleic acid
CFU-E	Colony-forming unit-erythroid
CSF	Colony-stimulating factor
DAG	Diacylglycerol
DNA	Deoxynucleic acid
DPPG	1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol
E-cad	E-cadherin
EDTA	Ethylenediamine-tetra acetic acid
EGTA	O,O'-Bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetra acetic acid
Epo	Erythropoietin
Fc	Fragment crystallisable
FCS	Fetal calf serum
Flt3/flk2	Fetal liver tyrosine kinase receptor

G418	Geneticin
G-CSF	Granulocyte colony-stimulating factor
GF	Growth factor
GFR	Growth factor receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HMW	High molecular weight
ICAM	Intercellular adhesion molecule
ICE	Interleukin-1β-converting enzyme
Ig	Immunoglobulin
IL	Interleukin
MAC	Membrane attack complex
mBPA	Membrane-bound burst promoting activity
M-CSF	Macrophage colony-stimulating factor
M-CSFR	Macrophage colony-stimulating factor receptor
MHC	major histocompatibility complex
mRNA	Messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Multi-CSF	Multilineage colony-stimulating factor
NGF	Nerve growth factor
NK	Natural killer

IP_3	Inositol triphosphate
pADPRp	poly(ADP-ribose) polymerase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PI	Propidium iodide
PIP	Phosphatidylinositol 4, 5-bisphosphate
PMSF	phenylmethylsulfonyl fluoride
P/S	penicillin/streptomycin
RNA	Ribonucleic acid
PKC	Protein kinase C
SCF	Stem cell factor
SCFR	Stem cell factor receptor
SDS	Sodium dodecyl sulphate
SM	Sphingomyelin
SMase	Sphingomyelinase
TBE	Tris borate EDTA buffer
TBS-T	Tris-buffered saline-Tween
TEMED	Tetramethylethylene diamine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Tris	2-amino-2-hydroxymethyl-1, 3-propanediol
UV	Ultraviolet

PUBLICATIONS, ABSTRACTS AND AWARDS ARISING FROM THIS WORK

Publications:

1. Dainiak N and Albanese, J. (1996). Effects of radiation on the plasma membrane and expression of membrane bound hematopoietic cytokines. *In* Gene Technology, *In* Analysis of Malignant and Inherited Human Disease Related to Development, NATO, ASI Series, A. Zander, editor, 1997. pp 405-418.
2. Albanese J. and Dainiak N. (1997). Early plasma membrane events occurring in ultraviolet-B-induced apoptosis. Stem Cell 15(suppl 2):49-57.
3. Albanese J. Meterissian S, Kontogiannea M, Hand A, Dubreuil C, Sorba, S. and Dainiak N. (1998). Bioactive Fas-ligand(FASL) and Fas receptor are shed on plasma membrane-derived vesicles in vitro. Blood 91: 3862-3874.

ABSTRACTS:

1. Albanese J., Dubreuil, C., Dainiak, N. (1997). Presentation of bioactive Fas antigen and ligand (FASL) on plasma membranes and shed vesicles. 26th Annual Meeting of the International Society for Experimental Hematology. Exp. Hematol. 25:Abstract No. 384.
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healthy individuals and myelodysplastic syndrome. Blood 88(suppl 1): Abstract No. 372.

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AWARDS:

1. Trainee Investigator Award. 1995. The American Federation For Clinical Research (AFCR). San Diego (USA) Conference.

2. New Investigator Award. 1997. The International Society For Experimental Hematology (ISEH). Cannes (France) Conference.

Introduction

Section 1: Plasma Membrane Structure

The plasma membrane is a highly organized structure, consisting of a complex array of lipids, proteins, glycolipids and glycoproteins which extends over the surface of the cell in the form of a lipid bilayer. Its organization is designed to facilitate it as a semi-permeable barrier between the cytoplasm and the extracellular environment, permitting molecules such as water to readily diffuse into the cell's interior while allowing charged ions (e. g., Na^{+}, K^{+} and Ca^{2+}) and nutrient molecules (e. g., glucose) to traverse the bilayer only via transmembrane carrier molecules. In addition, signals (e. g., proliferative, apoptotic, migration, recognition and other cues) are often transduced across the bilayer via receptor molecules that span the plasma membrane thus bypassing the necessity of transmembrane passage of the signaling molecule.

Section 1.1: Components Of The Plasma Membrane

1.1.1 Polar Lipids

Lipids are water-insoluble organic molecules that can be extracted from cells with nonpolar solvents such as chloroform, alcohol or ether[1]. Polar lipids are the major components of the plasma membrane and although insoluble in aqueous environment, they contain a

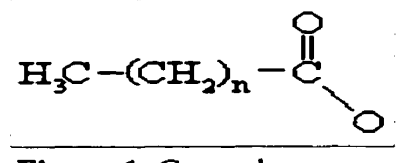


Figure 1. General structure of a fatty acid

hydrophillic moiety in their structure[2]. Of these, the simplest are fatty acids which are

composed of a hydrocarbon tail of varying length and one carboxyl group (Figure 1). Free fatty acids are present in the plasma membrane at a very low level. However, significantly increased levels are generated during pathological conditions[3] (e. g., Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease). The tendency of fatty acids to form micelles (instead of bilayers) has been shown to induce plasma membrane instability and cell injury[4].

Phospholipids constitute the major class of lipids of plasma membrane. Phospholipids contain a glycerol (phosphoglycerides) or sphingosine (phosphosphingolipids) backbone. In the case of phosphoglycerides, one of the glycerol hydroxyl groups is linked to a polar phosphate-containing moiety and the other two (C1 and C2) are esterified to the

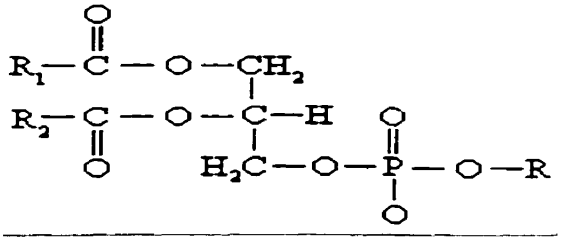


Figure 2. General structure of phosphoglycerides

carboxyl groups of two fatty acid chains (figure 2). The fatty acid chains vary in length (between 14 and 24 carbon atoms) and often, in phosphoglycerides, one chain is unsaturated, having one or more double bonds, while the other fatty acid molecule has none (saturated). The double bond produces a kink in the hydrocarbon chain which prevents fatty acid molecules from packing against each other, thus increasing the plasma membrane fluidity[5]. The predominating phosphoglycerides in mammalian cells are phosphatidylcholine,

phosphatidylserine and phosphatidylethanolamine. These diacylglycerides linked via a phosphodiester bond to choline, serine and ethanolamine moieties, respectively, at the C3 position of the glycerol backbone. Interestingly, diacylglycerides are asymmetrically distributed within the plane of the lipid bilayer. Phosphatidylethanolamine and phosphatidylserine are primarily concentrated on the inner leaflet of the membrane bilayer, while phosphatidylcholine is on the extracellular face of the membrane. Only phosphatidylserine, owing to its head group, possesses a net negative charge; a feature that is believed to be essential in mobilizing protein kinase C (PKC), an upstream initiator of many signaling pathways, from the cytoplasm to the plasma membrane[6,7]. In addition, accumulating evidence suggests that upon induction of programmed cell death (i. e., apoptosis), phosphatidylserine translocates to the outer leaflet of the plasma membrane where it serves as a signal for phagocytosis by the macrophage[8,9]. Phagocytosis of dying spermatogenic cells by Sertoli cells in primary culture also appears to be dependent on the

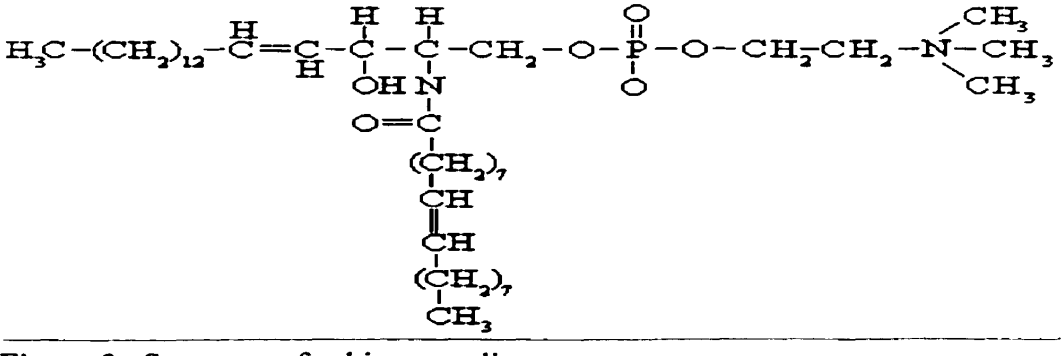


Figure 3. Structure of sphingomyelin

externalization of phosphatidylserine[10]. Hence, the difference in lipid composition between the inner and outer surface of the plasma membrane has a functional significance for

intercellular events (i. e., transmembrane signaling) as well as for intracellular recognition. Phosphosphingolipids are another major class of plasma membrane lipids; these lipids contain sphingosine, an amide-linked fatty acid group and polar group located to the C1 position through a phosphoester linkage. Sphingomyelin (SM) is a representative of this class of lipids (see figure 3). SM has choline as its polar head group and is localized to the outer leaflet of the plasma membrane.

Glycolipids, the most asymmetrically distributed of the plasma membrane lipids, are also sphingosine based and are present solely on the extracellular face of the lipid bilayer. As in the case of SM, the amino group of sphingosine is acylated. However, in contrast to phosphosphingolipids, these glycolipids are associated with a single (i. e., cerebrosides) or multiple (i. e., gangliosides) carbohydrate molecules instead of choline, ethanolamine or serine. Moreover, the link to the C1 hydroxyl group of sphingosine backbone occurs via a glycosidic bond, rather then through a phosphodiester bond[11]. Localization to the exterior face of the lipid bilayer may permit glycolipids to function as cell surface receptors[12]. Recent evidence suggests that glycosphingolipids participate in cell growth[13,14], differentiation[15], and transformation[16].

The functional aspects of phosphoglycerides and phosphosphingolipids are highlighted by their direct participation in transmembrane signal transduction, further emphasizing the notion that lipid provide more than structural organization of the plasma membrane. For example, in the inositol triphosphate (IP_3) signaling pathway, the enzymatic hydrolysis of the phosphodiester bond linking a phosphorylated inositol polar head group to the acylated glycerol moiety of phosphatidylinositol 4,5-bisphosphate (PIP_2), by activated

phospholipase C, generates (IP_3) and diacylglycerol (DAG). The action of IP leads to an increase in intracellular calcium concentration[17,18]. DAG induces the activation of protein kinase C by increasing its affinity for calcium[19]. In many cells, activation of PKC initiates a phosphorylation cascade culminating in cell activation and gene transcription[20]. In similar fashion to the formation of IP_3 and DAG, the activation of sphingomyelinase (SMase) triggered by the binding of a ligand (e. g., tumor necrosis factor α (TNFα) and interleukin 1 (IL-1)) to its cognate receptor leads to the hydrolysis of plasma membrane SM to generate ceramide and choline[21]. Ceramide acts as a second messenger that modulates the activity of several enzymes (PKCζ, ceramide-activated protein phosphate and ceramide-activated protein kinase) resulting in the activation of NF-κB and regulation of *c-myc*[22,23]. The ensuing response to the formation of ceramide is induction of cell differentiation[24], cell cycle arrest[21,25] or apoptosis[21,26]. That breakdown products of membrane lipids are key participants in transmembrane signal transduction emphasizes the concept that the plasma membrane does more then simply define the boundaries of a cell; its constituents play a vital role in critical events such as cell maturation, proliferation or cell death.

Section 1.1.2 Plasma membrane Proteins

Whereas the principle role of plasma membrane lipids is to establish a permeability barrier between the extracellular and intracellular environments, the dynamic aspect of the lipid bilayer is provided by plasma membrane proteins. Indeed, the amount and type of protein found on the cell surface membrane varies from cell type to cell type according to its function. For example, the plasma membrane of Schwaan cells, myelin, surrounding some nerve axons, is composed of approximately 20% protein and 80% lipids[27]. The high lipid content in myelin enables this membrane to serve as an electrical insulator for nerve axons,

allowing almost no current leaks across it[28]. In contrast, 52% of the plasma membrane mass in erythrocyte is protein. Two-dimensional separation employing isoelectric focusing and SDS-PAGE has revealed that the erythrocyte plasma membrane contains over 100 different proteins[29,30]. About 25% of total membrane protein is band 3, an anion transporter that catalyzes the exchange of HCO_3^- for Cl^- across the phospholipid bilayer, thus rendering it permeable to bicarbonate and chloride ions[31]. This anion exchange and consequently, band 3 is critical for the erythrocyte's raison d'être: the transport of CO_2 form the tissues and its exchange for O_2 in the lungs[32].

Protein associate with the plasma membrane in different ways and have been characterized as peripheral or integral based on relative strength of attachment[33]. Many proteins are bound to the phospholipid membrane either indirectly via electrostatic interactions with other proteins embedded in the bilayer (e. g., spectrin[34] and ankyrin[35], cytoskeletal proteins present in erythrocytes, are bound to the membrane through their interaction with the cytoplasmic domain of band 3) or directly by interactions with the charged head groups of the lipids forming the membrane (e. g., myelin basic protein[36] interacts with lipid polar head groups directly). Proteins bound to the plasma membrane by means of electrostatic associations can be extracted using mild treatments such as high salt solutions which disrupt ionic bonds; such proteins have been characterized as peripheral or extrinsic proteins.

Transmembrane proteins, those which interact with the hydrophobic core of the bilayer, are extracted only with strong detergents or solvents and are referred to as integral or intrinsic proteins. Proteins which traverse the plasma membrane, do so either once such as is the case for many cytokines and cytokine receptors (see below) or multiple times as is

the case for the β-adrenergic receptor which spans the membrane seven times[37].

The extracellular domains of transmembrane proteins (e. g., cytokine receptors) are often glycosylated. Carbohydrate moieties, usually, but not exclusively, *N*-acetylglucosamine or *N*-acetylgalactosamine, are linked to the protein via the amide of asparagine (N-glycosidic oligosaccharides) or through the hydroxyl groups of serine or threonine (O-glycosidic oligosaccharides)[38]. Mounting evidence suggests that extracellular carbohydrates on glycoproteins play an important role in intercellular recognition[39]. Indeed, given the different monosaccharides that can be joined to each other on any of the sugars' hydroxyl residues, and the extensive branching that is possible, the potential for structural diversity is clearly evident[40]. Such diversity is well suited to function in cell recognition processes[41]. In addition, it has been shown very recently that the presence of carbohydrate moieties on the extracellular region of the interleukin-1 receptor is important for binding of interleukin-1β to the receptor[42]. Similarly, only fully glycosylated natriuretic peptide receptors are able to bind natriuretic peptide suggesting that sugar residues on the extracellular domain of these receptors may be critical for receptor-ligand binding[43]. Whether carbohydrate molecules on the receptors directly interact with the ligand or contribute to the optimal conformation of the ligand binding site is not clear.

The intracellular portion of many cytokine receptors are often associated with kinase activities which serves to initiate signal transduction when activated by the binding of ligand. For instance, tyrosine kinase activity associated with the cytosolic domains of stem cell factor (c-Kit) and flt3/flk2 ligand receptors becomes activated following the binding of c-Kit and flt3/flk2 ligands to the extracellular domain of their respective receptors[44,45]. Both these ligands stimulate the growth of mouse[46] and human hematopoietic progenitor cells[46,47] and

may also trigger the proliferation of bile duct epithelial cells in mice[48]. Other cytokine receptors (i. e., interleukin-8 receptor) transduce extracellular signals across the plasma membrane via the interaction of their cytosolic domain with guanine nucleotide regulatory proteins (G proteins)[49]. The mechanisms involved in transmitting extracellular signals into the cell interior via G protein-coupled plasma membrane receptors form the basis of many excellent reviews (see [50,51,52]). Here, these examples illustrating basic structural differences between the extracellular receptor surface, critical for ligand binding, and the intracellular receptor surface involved in phosphorylation of cytosolic proteins or in G protein recognition and activation serve as a reminder that the orientation of transmembrane proteins within the lipid bilayer significantly contributes to the functional asymmetry of the plasma membrane. In the same vein, the orientation of ion channels in the plasma membrane allows for vectorial exchange of ions[53].

The transmembrane domain(s) of proteins crossing the lipid bilayer are composed non polar amino acids which adopt an α-helical conformation in order to maximize hydrogen bonding within the polypeptide backbone. Hydrophobic residues extend outward from the core of the α-helix and interact favorably with the hydrophobic milieu of the lipid bilayer. This conformation which allows the polar amide groups to hydrogen bond to each other and nonpolar residues to interact with the fatty acyl interior of the membrane minimizes the free energy for inserting the protein into the lipid bilayer[54] Finally, proteins can interact with the plasma membrane via a hydrophobic anchor consisting of a short terminal segment of nonpolar amino acids penetrating into the membrane[55]. In some cases, proteins are linked to the membrane via fatty acetylation in which myristic[56] or palmitic fatty acids[57], or

phosphatidylinositol[58] from covalent linkages to amino or sulfhydryl, or carboxyl groups, respectively.

Section 1.2: Radiation Effects on the Plasma Membrane

1.2.1 Direct and indirect action of radiation

Cell injury resulting from exposure to radiant energy (e. g., ultraviolet light or x-rays) occurs from either direct or indirect interaction of radiation with target organelles. In direct action, radiation is absorbed by atoms of target macromolecules resulting in the expulsion of electrons and rupture of covalent bonds. Alternatively, molecules may receive energy from a molecule originally ionized as a consequence of direct interaction with radiant energy, and become ionized themselves; this is referred to as indirect action of radiation. Since water is the principle component of eukaryotic cells, the latter action is important in biological systems. Hence, the accumulate effect of radiation on biological systems results from its interaction with water molecules and the subsequent generation of free radicals that diffuse throughout the cell and damage biomolecules[59].

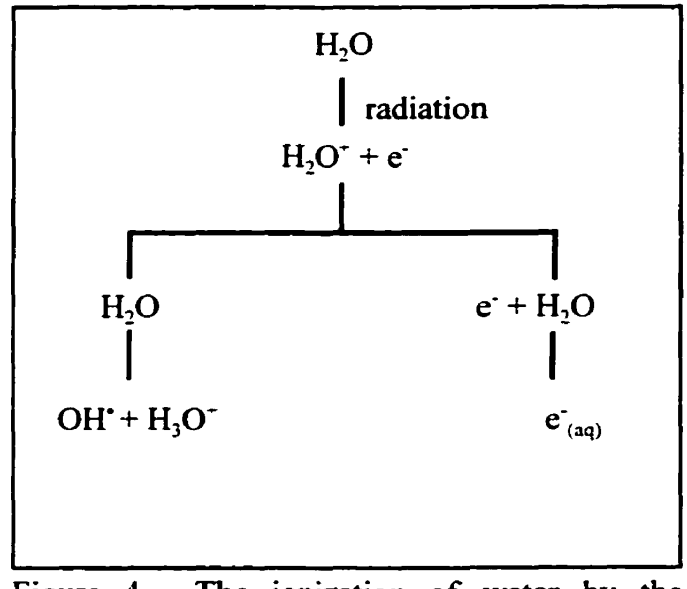


Figure 4. The ionization of water by the interaction with radiant energy generates hydroxyl radical ($OH^{\cdot}$) and the aqueous electron ($e^-_{(aq)}$)

The mechanism of water radiolysis has been extensively reviewed elsewhere[60]. As figure 4 illustrates, the first step in the ionization of water requires the absorption of radiant

energy. Radiation expels an electron (fast electron) from the water molecule and produces a H_2O^+ ion radical. The H_2O^+ species has an extremely short lifetime, and dissociates within 10^{-14} s to generate a highly reactive hydroxyl radical ($OH^\bullet$) and a hydronium ion (H_3O^+). The fast electron generated in the initiating step can interact with nearby water molecules to form H_2O^-, often referred to as a hydrated or aqueous electron and denoted as $e^-_{(aq)}$.

Additionally, the interaction of ionizing radiation with water may lead to a homolytic bond dissociation between the oxygen atom and one of the hydrogen atoms and generate a hydrogen radical ($H^\bullet$) as well as, a hydroxyl radical. This can be expressed as:

$$H_2O \xrightarrow{\text{Energy}} H^\bullet + OH^\bullet \qquad (1)$$

Hence, radiation interacts with water to generate three highly reactive species: hydroxyl radicals, hydrogen radicals and hydrated electrons. These undergo chemical reactions with cellular proteins, nucleic acids, lipids and carbohydrates which result in a final observed biological effect[61].

1.2.2 Formation of secondary radicals

The hydrated electron and the hydrogen radical generated by radiolysis of water can readily reduce molecular oxygen, yielding a superoxide radical and perhydroxyl radical, respectively. This is represented as in equations 2 and 3 below:

$$O_2 + e^-_{(aq)} \longrightarrow O_2^{-\bullet} \qquad (2)$$

$$O_2 + H^\bullet \longrightarrow HO_2^\bullet \qquad (3)$$

$$HO_2^\bullet \rightleftarrows O_2^{-\bullet} + H^+ \qquad (4)$$

The perhydroxyl radical is a weak acid (pK_a 4.8) and is deprotonated at physiological pH

(equation 4). The superoxide radical is predominant in the cell when generated as a secondary product of water radiolysis[62].

Contained in the phagosomes of macrophages and neutrophils, are superoxide radicals which fulfill an essential role as bactericidal agents[63]. Accumulating evidence has indicated that reactive oxygen species are generated in small quantities in response to the binding of cytokines to their receptors and are important in transmitting signals to the nucleus[63,64]. Despite the physiological roles of the superoxide anion, its chemical instability, like that of other active oxygen radicals (i.e, perhydroxyl radical, hydroxyl radical) cause oxidative damage to cellular biomolecules which include as DNA strand breaks[65], oxidative damage of proteins[66] and lipid peroxidation [67]. In human sperm cells, for example, lipid peroxidation results in plasma membrane damage leading to loss of motility. Storey (1997) reported that the extent of lipid peroxidation is directly proportional to levels of superoxide anion[68].

1.2.3 Lipid peroxidation of membranes

Polyunsaturated lipids such as those in the plasma membrane, possess double bonds between some of their carbon atoms that are susceptible to attack by oxygen radicals. Studies have indicated that in addition to ionizing radiation, ultraviolet light radiation also leads to a free-radical induced lipid peroxidation in model membranes[69]. Radicals generated as a consequence of water radiolysis act on the polyunsaturated fatty acids of cellular membranes and generate lipid peroxides[70]. A mechanism for the formation of lipid peroxides is given in figure 5.

The initiating event leading to lipid peroxidation is abstraction of the allylic hydrogen

from the polyunsaturated fatty acid by a hydroxyl radical. This generates a lipid radical which subsequently undergoes molecular rearrangement, forming the more stable conjugated lipid radical. A lipid peroxyl radical is formed as oxygen reacts with the conjugated lipid radical. Abstraction of a hydrogen atom from another lipid molecule generates a lipid

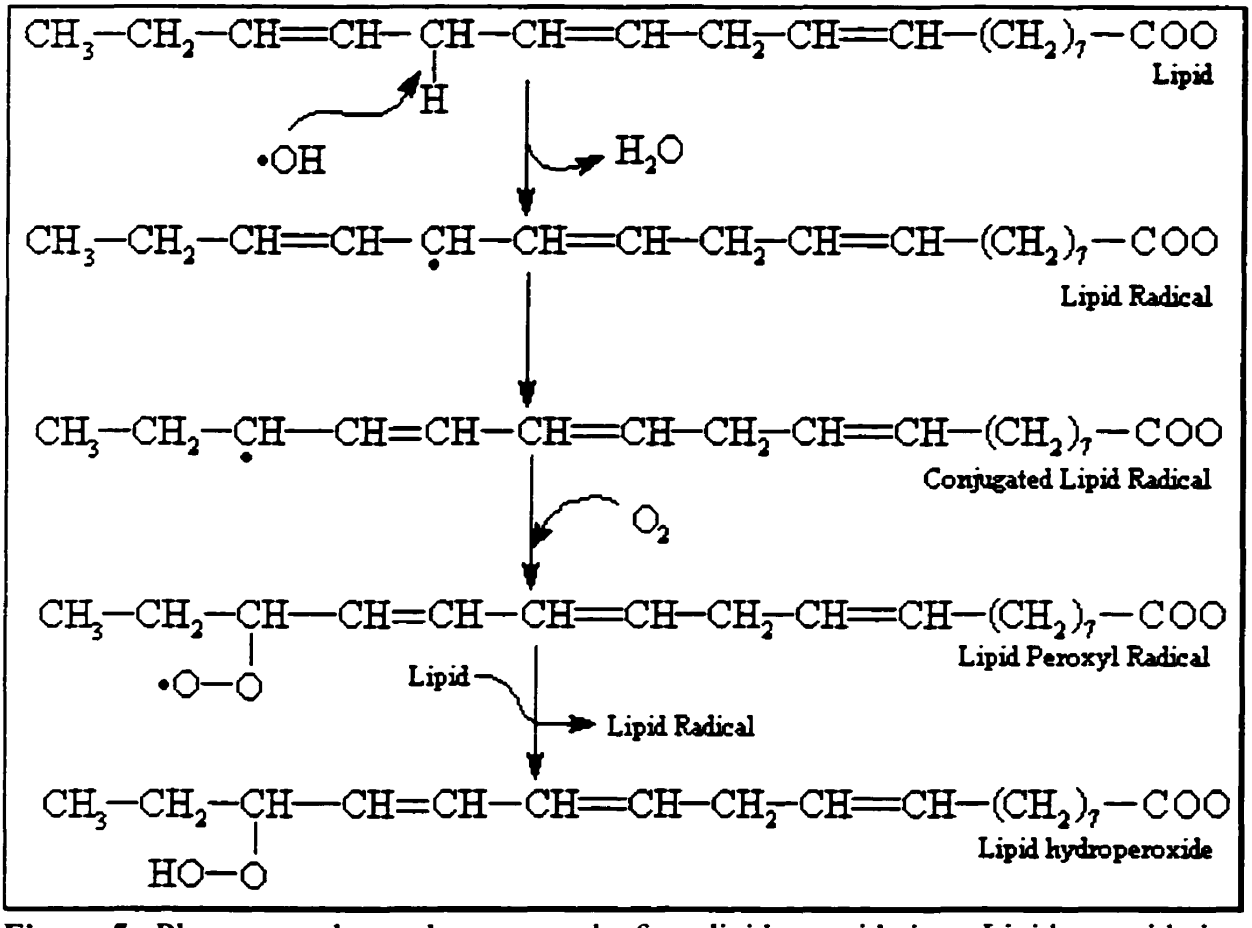


Figure 5. Plasma membrane damage results from lipid peroxidation. Lipid peroxidation is initiated by a hydroxyl radical which abstracts a hydrogen atom from polyunsaturated lipid molecules (lipid). This generates a lipid radical where electron shuffling leads to the formation of a conjugated lipid radical. Molecular oxygen adds to the conjugated lipid radical to form a lipid peroxyl radical which can, in turn abstracts a hydrogen atom from a nearby lipid molecule and produces a lipid hyrdoperoxide molecule and regenerates the lipid radical. This lipid radical can react with another lipid molecule and begin the cycle again. Hence the hydroxyl radical initiates a reaction that is self-perpetuating and results in the oxidative deterioration of polyunsaturated lipid molecules.

hydroperoxide and a new lipid radical. The lipid radical can then react with oxygen and a third lipid molecule and thus initiate an autocatalytic reaction which propagates the generation of lipid peroxides that ultimately, results in extensive plasma membrane damage. In this manner, lipid peroxides are continually formed, until lipid radicals taking part in the chain reaction are eliminated by radical-radical interactions[71].

1.2.4 Structural alterations of the plasma membrane as a consequence of radiation

Studies have shown that lipid hydroperoxides and lipid hydroperoxide breakdown products, including α, β unsaturated aldehydes such as malondialdehyde, alter plasma membrane fluidity, inducing a liquid crystal-to-gel phase transition[72]. Recently, Edimecheva et al. (1997), employed differential scanning calorimetry to observe that a liquid crystal-to-gel phase shift in liposomes from SM and 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol (DPPG) is induced by irradiation. As a result of the OH group at the β-position to the phosphoester bond in DPPG and to the amide bond in SM, DPPG and SM undergo a free radical induced fragmentation rather then peroxidation[73]. Accumulation of fragmentation products of DPPG and SM result in an increase in the temperature of the gel-to liquid crystalline phase transition[74]. To explain the physical interactions that lead to this phase transition, it has been suggested that the formation of polar products leads to an increase in dielectric constant within the hydrophobic core of the bilayer[71]. Additionally, as a consequence of increased polarity within the hydrophobic due to the formation of lipid hydroperoxides, lipid fatty acyl chains are induced to cross the bilayer center and interdigitate with adjacent hydrocarbon chains in the opposing monolayer. Concomitant with interdigitation, is an increase in membrane microviscosity due to a decreased thickness of the bilayer and enhanced ordering of the lipid chains[71,75,76]. Furthermore, radical-radical

interactions may result in the cross linking of lipid radicals and contribute to restricting the mobility of the hydrocarbon chains thus, rendering the plasma membrane more rigid[71,76,77].

Membrane lipid peroxidation results in increased plasma membrane permeability to small molecules[78] and ions[79]. It has also been shown, however, that many transport systems are aborted as the cell surface membrane becomes more rigid[80,81,82]. In view of the decreased fluidity exhibited by the plasma membrane as a result of lipid peroxidation, an increased permeability to molecules and ions appears surprising. According to Stark (1991), this paradox is reconciled by assuming that in addition to an overall liquid crystalline-to-gel phase transition of the plasma membrane which occurs as a consequence of lipid peroxidation, local protrusions are formed at defined sites on the membrane[71]. This assumption is supported by recent studies which have shown that an increase in plasma membrane microviscosity results in the formation of "blebs" or protrusions on the membrane surface[83,84,85]. In 1981, Chandra and Stefani reported that localized protrusions and pores are seen on the plasma membrane of lymphocytes as early as 15 minutes following exposure to X-rays[86]. It is likely that selective permeability of the plasma membrane is compromised by the creation of such pores; molecules are thus permitted to pass through these structures in a nonspecific manner. Ultimately, the inability of the plasma membrane to maintain ionic homeostasis results in cell death[87].

Section 1.3: Apoptosis

1.3.1 Occurrences and feature of apoptosis

In the early 1970s, Kerr, Wyllie and Currie used the term apoptosis (from the Greek for "falling off") to describe a pattern of programmed cell death which they distinguished from necrosis on the basis of morphology[88]. Despite their continued efforts to further

characterize the process, their reports were overlooked for many years. Today, it is widely accepted that apoptosis forms the cornerstone of tissue and organ development. For example, apoptosis is central for regulation of cell population densities, a parameter of tissue maintenance determined by the balance between cell proliferation and cell death[89]. Suppression of apoptosis in these circumstances leads to disorders of cell accumulation, not the least of which is cancer[90,91]. In contrast, acceleration of apoptosis results in tissue atrophy[89, 91], examples include degenerative diseases of the nervous system (Parkinson's disease, and Alzheimer's disease) and hematological disorders such as aplastic anemia, chronic neutropenia and myelodisplastic syndrome. Additional occurrences of apoptosis are presented in Table 1. The ubiquitous manifestations of apoptosis, in mammals as well as, in other organisms, suggest that the process is a genetically mediated form of cell death that is a fundamental characteristic of all cells. Interestingly, this phylogenetically conserved mode of cell death is triggered by a variety of stimuli which induce closely comparable structural changes. Indeed, the morphological attributes put forth by Kerr *et al.* (1972) to describe apoptosis still remain the best criteria by which the phenomenon is defined[88]. Specifically, in the early stages of apoptosis, dying cells shrink and the cytoplasm becomes dense. Despite complete condensation of the chromatin, cytoplasmic organelles and the plasma membrane, which shows extensive blebbing, remain intact. Accordingly, at this stage, despite losing their capacity to proliferate, dying cells remain impermeable to dyes such as trypan blue. As apoptosis progresses, there is a characteristic swelling and subsequent lysis of cytoplasmic organelles (mitochondria and endoplasmic reticulum). During late-stage apoptosis, the entire cell fragments into apoptotic bodies, which remain membrane-bound and *in vivo,* undergo phagocytosis by macrophage[92,93].

Typical of cells undergoing apoptosis is internucleosomal DNA degradation which occurs in the early-phase of the process[94]. Many reports have implicated calcium- and/or magnesium-dependent endonuclease(s) as the enzyme(s) responsible for DNA fragmentation[95,96,97]. Recent reports suggest that DNAse I and DNAse II are more likely candidates for the DNA-cleaving activity[98,99,100]. Regardless of their identities, the enzyme(s)

Table 1: Physiological And Pathological Conditions Associated With Apoptosis

Events	Examples/Comments	References
Programmed destruction of cells during embryogenesis	Death of astrocytes in the developing cerebellum of rats; Drosophila glial cells undergo apoptosis following commissural axon tracts formation.	101, 102
Hormone-dependent involution in the adult	Ovarian follicular atresia; epithelial cell death in mammary glands after weaning; endometrial epithelium cell death progressively increases during the menenstrual cycle	103, 104, 105
Cell death in tumors	Spontaneous regression of infantile myofibromatosis tumors; apoptosis also occurs in *growing* tumors	106, 107, 108
Death of immune cells	B and T cells undergo cell death following cytokine depletion; deletion of autoreactive T cells in the developing thymus	109
Pathological atrophy of hormone-dependent tissues	Castration results in prostatic atrophy; administration of glucocorticoids leads to lymphocyte deletion in the thymus.	110, 111
Pathological atrophy in parenchyma organs after duct obstruction	pancreatic duct obstruction leads to cell death of acinar cells; ureteral obstruction results in cell death in the renal tubule.	112, 113
Cell death induced by cytotoxic T and NK cells	NK cells and T lymphocytes induce fas-mediated apoptosis in target cells	114, 115, 116
Cell injury in certain viral diseases	HIV infected neural cell and measles-virus infected cells undergo apoptosis	117, 118, 119
Cell death produced by a variety of injurious stimuli	mild thermal injury, low dose UV and X-rays, cytotoxic anticancer agents and hypoxia induce apoptosis	120

associated with this type of DNA damage are specific for exposed sites between nucleosomes in the genome and as such, nuclear DNA of apoptotic cells analyzed by agarose gel electrophoresis reveals a characteristic ladder of DNA fragments consisting of 180-200 basepairs and multiples thereof.

Although still considered the most prevalent form of DNA damage, internucleosomal DNA fragmentation is not the only, nor the earliest pattern of DNA modification in cells undergoing apoptosis[121]. Until recently, it has been assumed that morphological changes in the nucleus (*i.e.*, chromatin condensation) is a reflection of internucleosomal endonucleolytic cleavage of DNA[122]; however, this assumption does not appear to extend to all cell types. For example, when C3H10T1/2 mouse embryonic cell are induced to undergo apoptosis and the nuclear DNA analyzed by alkaline agarose gel electrophoresis only *single* stranded fragments of *high* molecular weight (HMW) (~ 4 kbp) DNA are observed. The single stranded DNA fragments appear even though chromatin condensation occurs[123]. This finding suggests that apoptosis in C3H10T1/2 does not require internucleosomal DNA fragmentation and that ultrastructural changes observed in the nucleus may reflect DNA degradation resulting in HMW DNA fragments rather than the 180-200 bp integer fragments. This study was supported by Oberhammer *et al.* (1993), who demonstrated that in several epithelial cell lines undergoing apoptosis (as defined by morphological alterations) DNA fragments of 50 kbp were generated before the appearance of smaller 180-200 bp multimer oligonucleosomes. Moreover, in epithelial cells derived from human prostate carcinoma (DU-145), no DNA ladder was observed despite the fact that these cells exhibited classical apoptotic morphology[124]. The conclusion drawn by these investigators was that apoptosis is not dependent upon internucleosomal cleavage, but in cells which do fragment their DNA to 180 bp multimers, the appearance of larger fragments (50 and 300 kbp) is always a prior

event. Others have shown that chromatin condensation during cell death in oligodentrocytes is not accompanied by internucleosomal DNA fragmentation[125]. Fournel *et al.* (1995), have investigated the occurrence HMW DNA fragments or internucleosomal DNA fragments in human B cell leukemia lines undergoing apoptosis induced by treatment with etoposide (an inhibitor of topoisomerase II), ionomycin (a calcium ionophore) or by cross-linking of membrane immunoglobulins with anti-immunoglobulin antibodies[126]. Two types of apoptosis were identified: apoptosis resulting in small DNA fragments (integers of 180-200 bp) and apoptosis associated with generation of large DNA fragments (100-150 kbp). The stimuli used to induce apoptosis did not affect the size of the DNA fragments seen in individual cell lines, rather the type of apoptosis occurring in any one line appeared to be an intrinsic property of that cell line. Taken together, these reports suggest that in some cells undergoing apoptosis, DNA degradation proceeds in a stepwise fashion where the generation of HMW DNA fragments involving single strand cleavage precedes the formation of 180-200 bp multimer fragments. In other cells (*e.g.*, thymocytes), early DNA cleavage into large fragments is by-passed and DNA is degraded immediately into small DNA fragments producing a typical DNA ladder pattern when analyzed by agarose gel electrophoresis. Yet in other cells, internucleosomal DNA degradation has no bearing on the process of apoptotic cell death[126].

To complicate an already obscure phenomenon, it appears that in some cases the generation of HMW DNA fragments in dying cells does not necessarily indicate that the cells are undergoing death by apoptosis. Kataoka *et al.* (1995), used A23187, a calcium ionophore to induce cell death in two human leukemia cell lines[127]. Following treatment with A23187, the myeloid leukemia cell line, HL60 showed morphological alterations characteristic of apoptosis with the appearance of both, HMW DNA fragments and smaller

internucleosomal DNA fragments. The T lymphoblastic cell line, MOLT-4 also generated HMW DNA fragments. However, in contrast to HL60, MOLT-4 cells died of necrosis following treatment with the ionophore. Hence, it appears that HMW DNA fragmentation like, internucleosomal DNA fragmentation[128], is not specific to apoptosis.

In 1992, Collins *et al.*, proposed that internucleosomal DNA cleavage should not be used as the sole criterion for identifying apoptosis[128], it now appears that this cautionary note should be extended to include HMW DNA degradation.

1.3.2 Radiation induces apoptosis

Over the past century a large body of evidence has been accumulated and indicates that radiation kills cells via necrosis or by DNA double strand breaks which results in mitotic cell death[61]. Within the last 15 years, however, it has become clear that in some cases, the pattern DNA degradation that follows irradiation is characteristic of that produced by apoptosis, rather than necrosis[129,130]. Moreover, the early events which lead to cell death do not necessarily take place at the nuclear level. Instead, evidence suggests that radiation-induced apoptosis is triggered by changes occurring in plasma membrane level. Haimovitz-Friedman et al. (1994), reported that ionizing radiation triggers hydrolysis of plasma membrane sphingomyelin, resulting in production of ceramide and induction of apoptosis[131]. In their work, a ceramide analogue was shown to reverse phorbol ester inhibition of radiation-induced apoptosis and as such, these authors hypothesized that ceramide acts a second messenger in the radiation-induced apoptotic cascade. Previously, Ramakrishnan and coworkers had shown that pretreatment of murine thymocytes with a water-soluble analogue of vitamin E (Trolox) protects thymocytes from radiation-induce apoptosis[132]. Furthermore, Trolox completely blocked radiation-induced influx of calcium into the cells. It is believed

that an increase in intracellular calcium mediates the activation of enzymes, such as PKC and phospholipases, required for apoptosis[133,134]. These results suggest that radiation-induced apoptosis in these thymocytes is triggered by damage to the plasma membrane. Recently, this group performed a time course study on the protective effects of Trolox on radiation-induced apoptosis[135]. Incubation with Trolox only during, or immediately preceding irradiation was insufficient to protect MOLT4 cells from apoptosis. However, incubation with Trolox for 4h following radiation treatment was sufficient to completely block DNA fragmentation. Withdrawal of cells from Trolox prior to 4 hours progressively decreased its protective effect on the cells. The authors concluded that membrane-associated oxidations are responsible for radiation-induced apoptosis and DNA fragmentation in MOLT-4 cells and that the process leading to these events is triggered early after irradiation.

In support of the notion that radiation-induced membrane alterations are important in triggering cell death via apoptosis is the work reported by Nakajima and Yukawa (1996). It was shown that lipid peroxidation in hepatocytes following Υ-irradiation resulted in a translocation of PKC from the cytosol to the plasma membrane and that treatment with Trolox prevented PKC migration to the bilayer[136]. Taken together, these studies provide evidence for a mechanism(s) in which radiation initiates early events of apoptosis by modification (i.e., lipid peroxidation) of plasma membrane lipids. These alterations result in increased intracellular calcium levels which in turn, mediates the activation of PKC and phospholipases. These enzymes initiate downstream events that conclude DNA fragmentation, typical for apoptosis. Alternatively, radiation may trigger the activation of SMase generating ceramide which may then function as a second messenger stimulating a cascade of kinases that activate the apoptotic pathway. The mechanism by which radiation

stimulates SMase is as of yet, undefined.

Section 1.4: Role of growth factors in the regulation of tissue and organ homeostasis

1.4.1 General features of cytokines and their receptors

Normal development of tissues and organs hinges on careful regulation of cell proliferation, migration, differentiation and apoptosis. Cytokines have a central role in each of these processes. To better understand how growth factors (GFs) regulate tissue homeostasis, a brief overview of the principle characteristics of cytokines is beneficial. Hematopoietic cytokines will be presented as a paradigm for the biology of GFs.

Various cytokines are involved in proliferation and differentiation of blood cells. The principal GFs involved in hematopoiesis include the colony-stimulating factors (CSFs), named for their ability to induce the formation of specific hematopoietic lineages. Four CSFs have been identified: macrophage CSF (M-CSF), granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF) and multilineage CSF (Multi-CSF) or interleukin (IL) 3 (IL-3). Erythropoietin (Epo) induces terminal erythrocyte development and regulates erythropoiesis. Other GFs with hematopoietic activity include IL-1 IL-4, IL-5, IL-6, IL-7, IL-8, and IL-9, IL-11 and stem cell factor (SCF). Their site of action are identified in Table 2.

In vivo and *in vitro* studies have revealed nine recurrent archetypes hematopoietic GFs. (1) One theme that was recognized early in cytokine research is that actions of GFs are pleiotropic; that is, they elicit different biological activities from different target cells[137]. The availability of recombinant cytokines has confirmed this. For example, IL-5 activates T cells, induces immunoglobulin secretion and stimulates eosinophil production[138]. Why one molecule can exert a variety of effects on different cells is unclear, but it is possible that the

same receptor uses different intracellular signalling pathways in different cells[139]. (2) Also

Table 2: Principle cellular sources and targets of human hematopoietic growth factors

Factor	Cellular source	Cellular target
Erythropoietin	peritubular cells (kidney)	BFU-E and CFU-E
G-CSF	Macrophage, T cells, endothelial cells, mesothelial cells	Multipotential stem cells, granulocytes
GM-CSF	T cells, endothelial cells, mast cells, fibroblast, macrophage	Multipotential stem cells, mature neutrophils granulocytes/ monocytes
M-CSF	Fibroblasts, monocytes, endothelial cells	Multipotential stem cells, monocytes/macrophage
Multi-CSF	T cells, mast cells	Multipotential stem cells, mast cells
IL-1α and β	Macrophage, T and B cells, astrocytes, keratinocytes, epithelial cells, osteoblasts, fibroblasts,	Thymocytes, neutrophils, hepatocytes, chondrocytes, endothelial cells, macrophage, epidermal cells
IL-2	T cells	T and B cells, macrophage
IL-4	T cells, mast cells, bone marrow stromal cells	Multipotential stem cells, T and B cells, mast cells, macrophage,
IL-5	T cells	Eosinophils and B cells
IL-6	Fibroblasts, T cells, macrophage, endothelial cells, mast cells, keratinocytes	T and B cells, thymocytes, hepatocytes
IL-7	Thymic stromal cells	Pro-B, Pre-B, thymocytes, T cells
IL-8	Monocytes, endothellial cells, epithelial cells, fibroblasts, keratinocytes, hepatocytes	Neutrophils, basophils, T cells
IL-9	T cells	$CD4^+$ T cells, mast cells, erythroid progenitor
IL-10	T cells (T_H2 and T_H0), macrophage, B cells	Macrophage, mast cells, NK cells
IL-11	Stromal cells	Hematopoietic progenitor cells, B cells, mast cells, megakaryocytes
Flt3/Flk2 ligand	Stromal cells	Myeloid and monocytic cells, Pro-B, Pre-B cells
SCF	Fibroblasts, bone marrow stromal cells, Sertoli cells, endothelial cells	Mast cells, hematopoietic progenitor cells

true of GFs, is that in addition to inducing proliferation of hematopoietic precursors, they have the ability to increase functional activity of mature progeny of these precursor cells. An example is G-CSF, which induces proliferation of granulocyte progenitor cell and has a profound effect on the function of mature neutrophils causing their activation[140]. (3) A third aspect of GF action is the ability of cytokines to exert their activity directly or indirectly. Direct acting cytokines bind to receptors on the surface of the cells whose activity is to be altered. Epo, IL-3, G-CSF and M-CSF are example of GFs which interact directly with cells bearing receptors for the cytokine. In contrast, IL-1 acts by inducing auxiliary cells (*e.g.*, T cells, B cells, macrophage, fibroblasts) to produce other growth factors (*e.g.*, G-CSF) which then act directly on hematopoietic precursors. (4) Hematopoietic GFs and interleukins frequently exhibit synergy; that is their combined effects on cellular activity is greater than the additive effects of the individual cytokine. Thus, for example IL-3 and both G-CSF and GM-CSF work together in stimulating granulocyte and granulocyte/macrophage colony growth[141]. The mechanism by which synergy is achieved is still poorly understood, but it has been suggested that the interaction of one cytokine (in the above example, IL-3) with its receptor may prime a cell to become responsive to additional signals (G-CSF and GM-CSF). Alternatively, all three receptors may have to be occupied for the correct signal to be delivered[141]. (5) Colony-stimulating factors interact with other hematopoietic GFs at various stages of progenitor cell maturation in a hierarchal fashion[142]. Il-3 acts early in the differentiation series to induce self-renewal, proliferation and differentiation of pluripotent stem cells which will ultimately lead to the formation of all nonlymphoid blood cells. GM-CSF acts at a slightly later stage but also induces the formation of all nonlymphoid lineages.

M-CSF promotes the formation of monocytes while G-CSF promotes production of neutrophils, IL-5 eosinophils, and epo, the differentiation of red blood cells. Evidence suggests that IL-11 is an additional differentiation factor for megakaryocytes[143], and IL-9 for erythrocytes[144]. (6) Cytokine networks provide signal amplification circuits. IL-1 for example, acts in an autocrine fashion and enhances the expression of its own gene[145]. IL-1 can also be amplified in a paracrine fashion. Lindemann *et al.* (1989) have shown that GM-CSF induces production of IL-1 in neutrophils[146]; IL-1 induces the expression of GM-CSF in endothelial cells[147]. Thus, there is an amplification loop in which GM-CSF, induced by IL-1, induces IL-1.

(7) Hematopoietic GFs are encoded by genes that share structural and functional characteristics. Most cytokine genes consist of three or four introns and four or five exons and are located on chromosome 5. IL-3, IL-4, IL-5 and GM-CSF are positioned within 500 kbp of each other. M-CSF and IL-9 are also located on chromosome 5. Many GF gene transcripts contain an AU-rich motif which act to destabilize heterogenous mRNAs attached to them. This results in enhanced susceptibility for degradation of the GF transcript by ribonucleases. Predictably, stabilization of GF mRNA transcripts would result in an accumulation of the GF message. Bagby *et al.* (1989), have shown this to be the case for GM-CSF mRNA transcribed in endothelial and fibroblast cells in response to stimulation by IL-1[148]. (8) Structural and functional similarities are not limited to genes encoding GF; receptor for hematopoietic GF (GFR) also share structural and function similarities. Three major families of GFRs have been identified: (i) those belonging to immunoglobulin superfamily (*e. g.*, IL-1R), (ii) those with conserved cysteine residues in their extracellular

domain and a characteristic try-ser-X-try-ser domain immediately proximal to the membrane spanning region (*e. g.*, erythropoietin receptor) and (iii) and those with tyrosine kinase activity (*e. g.*, M-CSFR and SCFR). All three classes contain an extracellular domain, a membrane-spanning domain and an intracellular domain. However, GFR in examples (i) and (ii) lack a known catalytic domain. Another interesting feature of some GFR in classes (i) and (ii) above, is that they share peptide subunits with other receptors. For example, IL-5, GM-CSF and IL-3 have distinct α-chains which determines the GF specificity but share the same β-chain; heterodimerization of the two chains results in a high affinity binding receptor capable of signal transduction[149,150]. (9) Finally, structural abnormalities of hematopoietic growth factors or their receptors can result in abnormalities of hematopoiesis (see discussion below).

1.4.2 GFs provide a means for intercellular communication

Growth factors are generally regarded as soluble molecules secreted by a variety of cells (*e. g.*, lymphocytes, endothelium, fibroblasts, *etc.*) and have the capability to promote cell growth by engaging surface receptors on target cells often positioned a short distance away. In this type of intercellular communication, GFs diffuse from effector to target cells. However, the actions of GFs need not be restricted by solubility; many GFs have a membrane-bound form retained on the surface of the effector cell which activates cognate receptors via juxtacrine stimulation, a process necessitating direct and physical cell-cell interaction between GF on the effector-cell surface and its receptor on target cells[151]. For example, membrane-bound stem cell factor on stromal fibroblasts has been shown to stimulate proliferation of adhered normal megakaryocytes[152], megakaryoblastic leukemia

cells[153] and of the myeloid cell line, MO7e[154]. In some cases, GFs acting in juxtacrine fashion are more efficient at transducing activating signals to target cells than their soluble counterparts[155]. In the case of MO7e cells for example, membrane-bound SCF was found to be a more persistent activator of c-kit (SCF receptor) associated tyrosine kinase than the soluble form of SCF[154].

This observation illustrates two important advantages of juxtacrine stimulation over activation by soluble factors: first, immobilization of GFs on a membrane surface provides a general mechanism to concentrate factors and prevents their diffusion ensuring that GFs remain in reach only of adjacent cells. Second, membrane-bound GFs induce a stronger and longer lasting response than soluble GFs, possibly by preventing rapid down regulation of cell surface receptor expression on target cells[154].

Alternatively, GFs can be shed from the effector cell surface as constituents of microvesicles which then act as shuttles to carry signal molecules to target cells. It is now well established that both normal and neoplastic cells exfoliate or shed plasma membrane-derived vesicles bearing cell surface constituents[156,157,158,159]. Apart from its obvious role in reducing surface area-to-volume ratio of cells, increasing evidence suggests that exfoliation plays an important role in regulating tissue homeostasis. Cytokines such as macrophage-colony stimulating factor[160], membrane-bound burst promoting activity or mBPA[161,162], and flt3/flk2 ligand have been shown to be present on plasma membrane-derived vesicles where they induce proliferation and differentiation of bone marrow progenitors. Other molecules, potential regulators of cell growth, have also been detected on extracellular shed vesicles; these include ecto-protein kinase[163], fragment crystallisable (Fc) domain of antibodies[164],

class I and class II major histocompatibility (MHC) antigens[165,166] as well as transferrin receptor[167]. Likewise, molecules which inhibit cell cycle progression have also been found on shed vesicles. Recently, Dolo et al. demonstrated that transforming growth factor (TGF)-β released from human breast carcinoma cells on extracellular vesicles, specifically targets lymphocytes that recognize cell surface tumor antigens, and inhibits lymphocyte proliferation[168].

Hence the release of signaling molecules on the surface of microvesicles defines another mode of intercellular communication that is in essence, a compromise between the release of soluble but diffusible factors able to trigger cognate receptors on target cells not necessarily adjacent to the effector cell, and juxtacrine activity where long lasting stimulation can be achieved but only through direct and physical contact between effector and target cell. Growth factor-bearing microvesicles afford the freedom of *long* range communication while still maintaining GFs concentrated on a membrane surface and thus, restraining GF diffusion or dilution in the pericellular environment.

1.4.3 Exfoliation of tumor cell-surface constituents may play a role in cancer pathology

It has been suggested that the metastatic propensity of cancer cells may be enhanced by exfoliation. Studies showing that, in some cases, high metastatic potential cell lines shed higher levels of plasma membrane-derived vesicles relative to their low metastatic potential counterparts have strengthened this concept[169]. At the very least, shedding of glycoconjugates and tumor antigens from the surface of malignant cells may provide a mechanism by which these cells suppress antigen presentation[170], alter immune effector responses[171], and escape immune detection[172] thus prolonging cancer cell survival.

Of the nonimmunological consequences of cancer cell exfoliation, perhaps an important one with regards to metastasis, is the release of adhesion molecules, in particular E-cadherin (E-cad), integrin α4β1 and biliary glycoprotein (BGP). It has been observed that expression of BGP molecules, human homologues of the rat cell adhesion molecule (CAM) are down regulated in human colon carcinoma cells[173]. Recent studies have shown that cancer cells transfected with BGP-1 cDNA, when injected into syngeneic mice, fail to establish tumors in their new hosts[174]. Similar data are obtained with regards to cell surface expression of α4β1, a member of the integrin β1 subfamily of adhesion molecules. Melanoma cells expressing α4β1 molecules display homotypic intercellular adhesion that is disrupted when the cells are treated with an antibody against the integrin[175]. When assayed in vitro, melanoma cells treated with anti-integrin antibody display enhanced invasive capabilities compared to melanoma cells treated with an isotype matched antibody. Moreover, the ability of a highly metastatic melanoma cell line which does not express functional α4β1 adhesion molecules, to establish pulmonary metastases can be suppressed by transfecting the cells with cDNA encoding for the integrin molecules. Although the mechanisms by which integrin α4β1 and BGP prevent metastasis remain ill-defined, it can be argued that facilitated cellular migration exhibited by some cancer cells lines results from loss of intercellular contacts mediated by surface α4β1 and BGP molecules. Corroborating this hypothesis are studies performed by Byers et al. (1995), demonstrating that tumor cells which do not express E-cad, or cells that do express the adhesion molecule but which is weakly linked to the cytoskeleton, more readily detach from a tumor mass when exposed to low shear forces relative to E-cad positive cells[176]. No expression or expression of defective

E-cad molecules on cancer cells may provide a mechanism for weakening cell-cell contacts thus allowing cells to detach from tumors.

Release of cancer cells from a primary tumor mass is an unconditional requirement for metastasis. Exfoliation can contribute to the metastatic cascade by providing a pathway by which cancer cells deplete levels of adhesion molecules from their cell surface, thereby compromising the intercellular adhesive integrity of a tumor. A recent report demonstrating the presence of integrins on plasma membrane-derived shed vesicles from human breast carcinoma cells lends support to this hypothesis[177].

1.4.4 Mechanism of Shedding

Much of our knowledge concerning exfoliation is the result of work performed in the 1970s. In the past 15 year relatively few reports have been published on the topic and this, despite findings suggesting the importance of extracellular vesiculation in normal and malignant cell development (reviewed in 158 and 178). Particularly crude is our understanding of the mechanism(s) involved in shedding; however, several lines of evidence suggest that enhanced proteolysis and, lipolysis mediated by phospholipases, at the cell surface as well as disruption of cytoskeletal components and the destabilization of plasma membrane by ion fluxes are involved in mediating the release of plasma membrane fragments from the cell surface.

That ion influxes are important mediators of exfoliation was first suggested by the observation that activated cells shed more vesicles than resting cells. Later studies, where cells treated with calcium ionophores were shown to shed more than untreated cells, implicated calcium as a potential regulator of membrane-vesicle formation and shedding[179].

The importance of calcium in membrane shedding was further emphasized in publications by Morgan et al. (1985 and 1987), who demonstrated that release of membrane attack complex (MAC) on shed vesicles from the surface of neutrophils was preceded by an increase in intracellular calcium ($[Ca^{2+}]_i$) levels followed by its return to basal levels. When the rise of $[Ca^{2+}]_i$ was inhibited, shedding of MAC was eliminated resulting in an enhanced lytic susceptibility of the cells[180,181].

The importance of calcium as an intracellular regulator, influencing numerous cellular metabolic pathways is now well established[182]. Increased $[Ca^{2+}]_i$ may act directly or via calmodulin to mobilize phospholipases, proteases or cytoskeletal element, all of which can play a role in membrane exfoliation.

Suggestive evidence that proteases are involved in the shedding process was obtained from observations that cell surface molecules released by trypsin treatment and those released spontaneously into the medium of cells cultured in vitro, display a strikingly similar elution profile when subjected to ion exchange chromatography[183]. The authors concluded that "trypsin seems to perform its specific proteolytic function on certain surface sites by weakening their structure, thereby merely enhancing the normal process of release."

In the past two decades, mounting evidence has pointed to calpain, an intracellular calcium-dependent protease, as the principle enzyme responsible for cell membrane remodeling. Such evidence includes data demonstrating that anchor cytoskeletal elements to the plasma membrane are excellent substrates for calpain[184], as well as more direct indications demonstrating that calpain-induced hydrolysis of actin-binding protein and decreased association of actin with membrane glycoprotein is accompanied by increased

shedding of cell surface-associated procoagulant activity on vesicles derived from the plasma membrane of platelets[185,186]. Interestingly, studies by Raz and Geiger, had previously demonstrated that actin and vinculin are prominently associated with the plasma membrane of poorly metastatic cells, whereas highly metastatic cells demonstrated fewer contacts between these microfilament molecules and the plasma membrane[187]. These data, as well as other data demonstrating that highly metastatic cell lines shed more than their nonmetastatic counterparts[169] supports the hypothesis that dissociation of the cytoskeleton from its membrane attachment sites may play a critical role in the shedding process. Recently, it has been reported that the transition from nonmetastatic to metastatic human colon cancer cells associated with down-regulation of α-catenin, an actin-binding molecule which links E-cad to the cytoskeleton[188]. Whether disrupted cytoskeleton-plasma membrane contacts seen in cancer cells are the result of mutations in genes coding for cytoskeletal elements or the result of enhance calcium-dependant protease activity is not known. Nonetheless, it is increasingly apparent that in normal cells, calpain plays a pivotal role in exfoliation.

Although phospholipases are believed to be involved in the shedding process, their role remains obscure; it is well established, however, that they are required in fusion events[189]. As vesicles form and before they are shed from the cell surface, fusion must take place at the base of the newly forming vesicles between the two apposing sections of membrane. It has been postulated that phospholipases may mediate fusion by generating lysophospholipids, which exhibit detergent-like properties and allow free interaction between membrane structures.

1.4.5 GFs regulate apoptosis

Evidence that apoptosis is involved in the regulation of tissue size was initially provided by two studies performed by Columbano and coworkers. In the first of these studies, Columbano *et al.* (1985), initiated hepatic cellular proliferation causing liver enlargement in rats by a single intravenous injection of lead nitrate[190]. Histological examination of involuting livers sections revealed the presence of apoptotic bodies suggesting ongoing apoptosis. Apoptosis was found to be markedly increased at 5 days after treatment, a time period when the liver was already regressing while little apoptotic activity was shown during the first two days following treatment a time point when mitotic index reached its maximum, or at 15 days, when the liver had returned to control values. In a second study, Ledda-Columbano *et al.* (1989) demonstrated that renal hyperplasia resulting from a single intravenous injection of lead nitrate is accompanied by extensive apoptotic cell death which becomes attenuated as the organ regresses to its original size[191]. Recently it has been shown that regression of hypertrophied rabbit bladders involves apoptosis[192]. These results support the hypothesis that apoptosis is involved in the regulation of organ size. The question then arises, what regulates apoptosis in normally developing tissues?

Several lines of evidence have implicated GF as key regulatory determinants of apoptosis. Interleukin-2 dependent T cells undergo apoptosis when cultured in the absence of growth factor[193]. Another study demonstrated that cell death of hematopoietic precursor upon withdrawal of the relevant CSF is due to apoptosis, indicating that CSFs promote cell survival by suppression of the process of apoptosis[194]. Yu *et al.* (1993), described the importance of the hematopoietic cytokines, epo, IL-3, and SCF, in maintaining a homeostatic

balance of erythropoiesis and apoptosis in liver during ontogeny by determining the effects of these GFs on hematopoiesis by (a) measuring colony formation and hemoglobin synthesis in cultured fetal mouse livers and (b) ascertaining the protection from apoptosis afforded by these cytokines[195]. Their results showed that cells of the developing hematopoietic system not only require cytokines for proliferation and differentiation, but they have an initial and absolute requirement of them for protection from apoptosis. Previously, Koury *et al.* (1990), had shown that in the absence of erythropoietin, erythroid progenitor cells accumulated DNA cleavage fragments characteristic of those found in apoptosis by 2 to 4 hours and began dying by 16 hours[196]. In the presence of erythropoietin, the progenitor cells survived and differentiated into reticulocytes. The conclusion reached by these investigators was that apoptosis is a major component of normal erythropoiesis, and erythropoietin controls erythrocyte production by preventing apoptosis in erythroid progenitor cells. In a recent study, Diamond-Blackfan anemia was characterized as a disorder in which increased sensitivity of erythroid progenitor and precursors to epo deprivation results in accelerated apoptosis culminating in erythroid failure[197]. Together, these studies indicate that GF preserve cell viability by preventing apoptosis. A corollary of this notion is that hyperplasia is governed by availability of GF, and that cell accumulation disorders in tissues can be controlled by preventing growth regulators from exceeding normal levels.

1.4.6 GFs regulate apoptosis by modulating proto-oncogene expression

In the examples discussed above, as well as in other instances[198,199], it is apparent that cell survival is dependant on the presence of GFs and that their withdrawal leads to apoptosis. This phenomenon has been termed *growth factor addiction.* These observations

notwithstanding, it has been shown that withdrawal of a GF simply halts cell division without apoptosis. Careful scrutiny of the different responses of cells to GF withdrawal has implicated the proto-oncogene, *c-myc* as the key factor. As an example, the myeloid cell line 32D, transfected with *c-myc* undergoes apoptosis upon IL-3 withdrawal, whereas 32D cells not expressing the proto-oncogene exhibited cell cycle arrest at G1 phase and suppressed apoptosis[200]. In an earlier study, expression of c-myc in fibroblasts was associated with an increased incidence of mitosis, but incidence of single cell death by apoptosis was higher[201]. Similarly, overexpression of v-myc sensitizes preneoplastic bursal lymphoblasts to induction of cell death[202]. Interestingly, apoptosis was initiated when cell-cell contacts between lymphoid cells and avian bursa stromal cell layer in short term cultures were disrupted by mechanical dispersion. Given that many growth factors are cell membrane-bound (on the surface stromal cells), it is plausible that mechanical dispersion-induced apoptosis resulted from depriving lymphoid cells of GFs. In this situation, expression of *v-myc* induced a state of GF addiction. Clearly, c-Myc is an important player in triggering apoptosis in some cells. In other cells, *c-myc* expression is required for cell survival. In B lymphomas, for example, down regulation of *c-myc* expression results in cell death upon surface immunoglobin cross-linking[203]. Recently, Fischer *et al.* (1994), have reported that stabilization of c-Myc protects B lymphoma cells from anti-μ-induced apoptosis[204].

These studies have provided the ground-work for a model which speculates on how GFs prevent cell death (apoptosis) in cells expressing c-Myc. Advanced by Bissonnette and co-workers in an attempt to explain how homeostasis is maintained in normal tissues, the *Two Signal: Death/Survival Model* proposes that quiescent cells are induced to enter the cell

cycle as a consequence of *c-myc* expression; having departed from the resting phase cells proliferate or undergo apoptosis with equal probability unless acted upon by GFs[205]. Hence, whether a cell survives or dies during *c-myc* expression hinges on the presence or absence of GFs. In the absence of GFs, the default pathway is apoptosis driven by c-Myc. In the presence of GFs, apoptosis is blocked and a series of events are initiated that lead to proliferation and differentiation of the target cell.

The mechanism by which survival signals are transduced from GF receptors on the cell surface to the cytoplasmic environment and ultimately, the nucleus is not fully understood. Evidence suggests that calcium ions, GTP (guanosine triphosphate), cyclic AMP (adenosine monophosphate), phospholipids and protein kinases are involved. Through these pathways, GFs activate DNA binding proteins which bind to cytokine responsive elements leading to gene transcription. *Bcl-2* proto-oncogene (see below), has been shown to be transcribed in the neural cells in the presence of TGF-β. Prehn *et al.* (1994), have shown that hematopoietic growth factors, IL-3, Epo and bryostatin-1, a macrocyclic lactone natural product and potent activator of PKC, suppress apoptosis and induce the rapid serine phosphorylation of Bc1-2α, an isoform of Bcl-2[206]. These investigators propose that PKC phosphorylates Bcl-2 and by doing-so, enhancing the functional interaction between Bcl-2α and Bax (see below), consequently, apoptosis is suppressed. This possibility has yet to be confirmed.

In the same vein, genetic and biochemical studies have demonstrated that ras proteins transduce proliferation signals from receptor tyrosine kinases by switching from the inactive GDP-bound state to the active GTP-bound state[207]. The signal then proceeds to raf-1, then

Map kinase kinase, ultimately, resulting in activation of Map kinase[208]. The activation of Map kinase, in itself does not appear to provide a survival signal capable of blocking cell death, however, it is required for normal growth of hematopoietic progenitors (CD34+), as assessed by colony development *in vitro*[209]. Studies have shown that there is an accumulation of active ras-GTP following signalling through numerous GF receptors, including IL-2, IL-3, IL-6 and GM-CSF[210,211]. It is likely GFs activate Map kinase (via GTP-ras), which, in turn, phosphorylates transcription activators (c-Myc and c-Jun) in the nucleus, and trigger proliferation. Interestingly, it has been shown that IL-3 transduces a proliferation signal by activating PKC which directly phosphorylates raf-1. Taken together these reports suggest that PKC is a branch point promoting cell survival by (1) curtailing cellular apoptosis (phosphorylation of Bcl-2) and (2) activating raf-1 and ultimately transcription activators. Very recently, studies by Billadeau *et al.* (1995), have shown that the introduction of cDNA encoding for a constitutively activate N-ras protein into an IL-6-dependent myeloma cell line ANBL6 imparts IL-6-independence accompanied by suppression of apoptosis to the cell line[212]. Billadeau and coworkers found no changes in levels of Bcl-2 protein expressed in parent and transfected cell lines to account for increased suppression of apoptosis in mutant *ras*-expressing cells. Findings of May *et al.* (1994) regarding the role of phosphorylated Bcl-2 and suppression of apoptosis would suggest the possibility that ras activates a kinase which phosphorylates Bcl-2 resulting in reduced apoptosis.

1.4.7 Regulation of Apoptosis by Oncogenes

Early in the development of the nematode *Caenorhabditis elegans*, 1090 cells are produced and of these 131 undergo cell death via apoptosis (see [213] and refs. within). Through genetic

analysis investigators have shown that 3 of the 14 genes linked to apoptosis are critically involved in regulating the process[214]. *Ced-3* and *ced-4* must be functional for apoptosis to occur, while *ced-9* acts as an inhibitor of cell death and protects cells not destined to die. Interestingly, is the demonstration that *ced-9* has significant homology with *bcl-2*, a mammalian oncogene that acts to inhibit cell death in B cell lymphomas[215]. This finding suggests that the pathways which regulate apoptosis in *C. elegans* may be similar to those regulating cell death in mammals. In fact, expression of the human bcl-2 gene in *C. elegans* reduced the number of programmed cell deaths.

The *bcl-2* gene was originally identified in follicular lymphoma cells which revealed a characteristic t(14;18) chromosomal translocation[216]. This translocation moves the *bcl-2* proto-oncogene from its mapped position at 18q21 to the immunoglobulin heavy chain locus at 14q32. The resulting cytogenetic aberration leads to overexpression of *bcl-2* and accumulation of the 25 kd Bcl-2 transmembrane protein in the mitochondria, endoplasmic reticulum and the nuclear envelope.

The mechanism by which Bcl-2 inhibits cell death remains ill defined. There is evidence to suggest that the protein may act as an antioxidant, minimizing damage mediated by oxygen radicals[217,218]. In this scenario, results published by Nguyen *et al.* (1994) suggest that membrane anchoring mediated by a COOH-terminal hydrophobic tail of Bcl-2 is required for its full activity[219]. When these investigators deleted the membrane binding signal sequence of the protein, rendering Bcl-2 cytosolic, its ability to prevent apoptotic death in human KB cells was impaired. Replacing the anchor sequence restored Bcl-2's ability to prevent apoptosis. These results are consistent with a model in which the transmembrane

segment contributes to the function of Bcl-2 by targeting and anchoring the protein to strategic membrane locations in the cell. However, another report by Borner *et al.* (1994), suggests that membrane attachment of Bcl-2 attributed to the COOH terminal signal sequence is not critical for its activity, rather two conserved motifs (S-N and S-II) at the NH_2 and COOH terminus of Bcl-2 mediate the anti apoptotic activity[220]. Steinman (1995), has proposed that Bcl-2 does not function as an antioxidant but instead, Bcl-2 appears to influence levels of reactive oxygen intermediates that induce endogenous cellular antioxidants[221].

Kane *et al.* (1995), reported that bcl-2 inhibits necrosis of neural cells induced by glutathione depletion[222]. The interpretation of their experiments was that bcl-2 does not inhibit the cellular death program directly; rather, bcl-2 modulates a cellular process that leads to apoptosis under some conditions but necrosis under others.

It is conceivable that the mode of action of Bcl-2 is not the same in every cell type and may be dictated by the interaction of Bcl-2 with other proteins that may or may not be expressed in a particular cell type. A recent publication implicates p23-R-Ras, a member of Ras superfamily of GTPases, for the induction of apoptosis in IL-3 deprived, R-Ras(38V)-producing 32D.3 and FL5.12 cells[223]. Cells transfected with *bcl-2* exhibited prolonged cell survival in the absence of IL-3. The conclusion drawn by the researchers was that R-Ras enhances the activity of a cell death pathway in growth factor-deprived cells and that Bcl-2 acts downstream of R-Ras to promote cell survival. Whether Bcl-2 interacts proteins of the r-ras pathway is not known. Several protein have been identified as co-participants of Bcl-2 in regulating apoptosis; these proteins share sequence homology with

Bcl-2 and include Bax (Bcl-2 associated X protein), Bcl-x_S Bcl-x_L and Bad[224,225,226]. Bax protein promotes cell death. Bax-mediated cell death is suppressed by the heterodimerization of Bcl-2 (or Bcl-x_L) with Bax[224,226,227]. Hence, cell survival depends on the ratio between Bax and Bcl-2; if Bax is in excess over Bcl-2, apoptosis predominates, conversely, if Bcl-2 is in excess over Bax, apoptosis is inhibited. In yeast cells, Bad, a protein with limited homology to Bcl-2, competes with Bax for Bcl-2 and Bcl-x_L, thereby releasing Bax and reversing the death repressor activity of Bcl-2 and Bcl-x_L[226]. Recently, Hanada *et. al.* (1995) have shown that at least three regions (termed domains A, B, C) of the Bcl-2 protein important for suppression of Bax-mediated cytotoxicity. Domains B and C are required for binding of Bcl-2 to Bax, whereas domain A, while not essential for Bax-binding activity, is essential for anti-apoptotic activity[228]. Like Bcl-x_L, Bcl-x_S is generated by alternate splicing of the *bcl-x* gene. Unlike the long form of the protein, Bcl-x_S accelerates the susceptibility of cells to undergo apoptotic cell death. Overexpression of Bcl-x_S in MCF-7 cell renders the cells susceptible to chemotherapy-induced apoptosis[229].

1.4.8 Role of proteases in the induction of apoptosis

As noted above, *ced-3* and *ced-4* are critical for the induction of apoptosis in *C. elegans*. Inactivation of either of these genes abrogates cell death in cells normally destined to die. While it has been deduced, from cDNA and genomic DNA clones that the *ced-4* protein has a mass of 63 kD and two possible calcium binding regions, little is known concerning its functional significance in apoptosis[230]. On the other hand, considerably more is known about the *ced-3* protein and its role in cell death. Structural analysis of the gene product has shown that it share sequence homology with the mammalian cysteine protease interleukin-1β-

converting enzyme (ICE)[231]. This enzyme is specific for the 33 kD precursor of IL-1β and cleaves the pro-molecule between Asp-117 and Val-118 to generate the 17 kD active form of the cytokine[232]. The observation that expression of *ced-3* is most pronounced during embryogenesis in *C. elegans*, a time when most apoptosis occurs, implicated the *ced-3* gene product, and by virtue of its homology to mammalian proteolytic enzyme, implicated ICE as *the* death protease[231]. This idea was further strengthened by experiments in which overexpression of *ced-3* in Rat-1 fibroblast cells[233] and of ICE[234] resulted in apoptotic cell death. More evidence that ICE may play a central role in the regulation of apoptosis comes from experiments showing that suppression of ICE inhibits apoptosis in human myeloid leukemia U937 cells[235], mammary epithelial cells[236] as well as, in neurons upon nerve growth factor deprivation.

Despite the compelling evidence suggesting that in mammalian cells, ICE may be the functional equivalent of the Ced-3 protein, Li *et al.* (1995), have reported that transgenic mice deficient in ICE, although unable to generate active IL-1β, show no apparent defects in apoptosis[237]. One interpretation of Li and coworkers' work is that another protease(s) is involved in regulating apoptosis in mammalian cells. There are several possible candidate enzymes, members of the ICE family of , which could function as the mammalian counterpart of Ced-3. Since the initial isolation of ICE form monocytes, other Asp-specific cysteine proteases with ICE homology have been described and linked to apoptosis; these include the mouse Nedd2 protease[238], its human homologue, ICH-1[239], CPP32/YAMA[240,241], TX/ICH-2[242], and MCH-2[243]. On the basis of structural similarities, ICE homologues identified in humans have been classified into three principle categories[244,245]. Table 3 lists

Table 3:Classification Of Human Proteases With ICE Homology

Category	Trivial Name	Common Name	Substrate
I	Caspase-1	ICE	pro-Il1β, caspase-3, -4 zymogens
	Caspase-4	ICErel-II, TX, ICH-2	caspase-1 zymogen,
	Caspase-5	ICErel-III, TY	unknown
II	Caspase-2	ICH-1	pADPRp
	Caspase-9	ICE-LAPS6	pADPRp
III	Caspase-3	CPP32, Yama, apopain	pADPRp, DNA-PK, SRE/BP, rho-GDI
	Caspase-6	Mch2	lamin A
	Caspase-7	Mch3, ICE-LAP3, CMH-1	pADPRp, caspase-6 zymogen, MEKK-1
	Caspase-8	FLICE, MACH, Mch5	zymogens of the all members of the ICE superfamily
	Caspase-9	ICE-LAP6, Mch6	pADPRp
	Caspase-10	Mch4	unknown

the ten members of the human ICE protease superfamily that have been published to date and their corresponding trivial names proposed by Alnemri et al., (1996)[244].

All members of the ICE superfamily cleave their substrates following aspartate residues. Furthermore, proteases belonging to this family exist as zymogens that in turn require cleavage at internal aspartate residues to generate the two-subunit active enzyme. As such, family members are capable of activating each other[246]. Recently it has been shown that all known ICE family members serve as substrates for caspase-8; this enzyme initiates the apoptotic cascade following the activation of Fas receptor (see below)[247].

Several lines of evidence have implicated the action of poly(ADP-ribose) polymerase (pADPRp) as key to induction of apoptosis[248,249]. The polymerase enzyme is a 116 kD nuclear protein which is specifically cleaved to produce an active 85 kD fragment[249] whose actions result in an eventual depletion of ATP and, as a consequence, cell death[250]. From this prospective, prICE, a protease resembling ICE, is a principle candidate as the pivotal Asp-specific cysteine protease regulating apoptosis. PrICE has been shown to cleave pADPRp at a tetrapeptide sequence YVAD, which is identical to one of the two ICE cleavage sites in pro-IL-1β[251]. Provocatively, prICE does not cleave pro-IL-1β[239,251]. The inhibition of prICE by the ICE inhibitor Ac-YVAD-CMK blocks the biochemical and morphological alterations suggesting that prICE is at the apex of the apoptotic pathway[252]. CPP32 is another putative cysteine protease whose gene was recently cloned from human Jurkat T-lymphocytes[240,252]. The catalytic region of CPP32 contains amino acid residues conserved in ICE-like proteases and that are required for substrate binding and catalysis. Expression of CPP32 in Sf9 insect cells results in apoptosis. This apoptotic activity of CPP32 and its high expression in lymphocytes suggest that CPP32 is an important mediator of apoptosis in the immune system[239,251]. Tewari *et al.* (1995) cloned a cDNA, which they designated as YAMA, with a sequence almost identical to the cDNA encoding CPP32[241]. These investigators further demonstrated that purified YAMA is pro-molecule that, upon activation is able to cleave pADPRp and generate the 85 kD apoptotic fragment. Cleavage of pADPRp could be inhibited by CrmA, a cowpox viral protein and an inhibitor of ICE[253]. Furthermore, CrmA blocked cleavage of pADPRp in cells undergoing apoptosis. These finding strongly support YAMA as an effector component of the mammalian cell death pathway and suggest

that CrmA blocks apoptosis by inhibiting YAMA.

While it has been suggested that apoptosis may result as a consequence of cellular ATP depletion following protein ribosylation as catalyzed by pADPRp[250], the mechanism by which proteolysis of this enzyme contributes to apoptosis remains to be elucidated. In opposition to lines of evidence suggesting that pADPRp activation promotes cell death, other studies have shown that activated pADPRp hinders the process[249, 317,254]. Accordingly, investigators have proposed that because pADPRp facilitates access of DNA repair enzymes to damaged DNA sites, its proteolytic degradation obviates attempts at DNA repair and thus, enhances apoptosis[254]. Another plausible explanation stems from the observation that some endonucleases are inhibited by ADP-ribosylation[255]. Degradation of pADPRp is required in order to prevent their ribosylation of endonucleases and consequently, allow their activation during apoptosis.

Other targets of the ICE superfamily of proteases include lamins, the 70kDal subunit of U1 small nuclear ribonucleoprotein, Alzheimer-associated presenilin 1 and presenilin 2 endonucleases, MEKK-1, histones and nucleolins[255,256,257].

The studies described above strongly implicate proteases in the process of apoptosis. What is less clear, however, is which proteases are activated during the process and in what sequence.

1.4.9 Fas receptor

Fas receptor (Fas) is a 48 kDal transmembrane protein that, like the TNF receptor (p55), transduces an apoptotic signal when cross-linked by antibody, or its natural ligand. Fas is a member of a superfamily of cell surface proteins which includes the TNF receptors

(p55 and p75), p57 nerve growth factor (NGF) receptor, CD27, CD30, CD40, OX40 and 4-1BB. Membership to this family is bestowed to intrinsic proteins whose extracellular domains show amino acid homology to those of TNF and NGF receptors; the extracellular domains of these proteins are characterized by the presence of several cysteine-rich motifs. Fas shows greatest resemblance to the p55 TNF receptor in which homology extends beyond the extracellular domain to include the cytoplasmic domain where, a stretch of amino acids forms the *death domain*. A deletion or a point mutation is this region is sufficient to abolish Fas-mediated signal transduction[258,259]. Two cytoplasmic proteins with death domains, FADD an RIP have been shown to interact directly with the death domain of Fas receptor. Mutations altering the death domain of FADD abolish its ability to bind to the Fas and prevents Fas-induced apoptotic signaling.

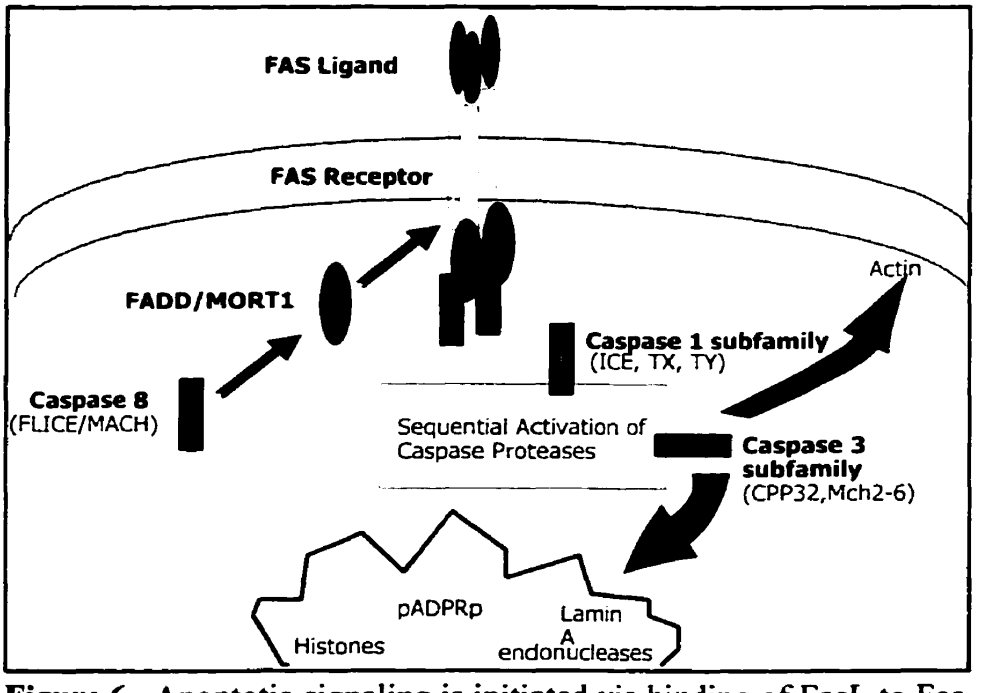


Figure 6. Apoptotic signaling is initiated via binding of FasL to Fas. This interaction induces the formation of a trimeric receptor complex that associates with FADD/MORT1. Caspase-8 is recruited to FADD/MORT1, where it may undergo autoproteolysis and become activated. Proteolytic activation of other members of the caspase superfamily by caspase-8 leads to degradation of many polypeptide species (e.g, pADPRp, lamins, actin) and possibly the activation of endonucleases that lead to the morphological features associated with apoptosis

Recently, several investigators have shown that FADD associates with caspase-8 (FLICE/MACH), a member of the ICE protease family (figure 6). The activated Fas receptor complex, induces proteolysis of the caspase-8 molecule, presumably activating the enzyme, which in turn triggers the ICE protease cascade[245].

It is well established that mutations of the Fas receptor, as well as Fas ligand (FasL), in humans and in mice, result in autoimmune and lymphoproliferative disorders resembling systematic lupus[250,260,261]. It has been suggested that expression of nonfunctional Fas receptor or FasL hinders activation-induced cell death (ACID), a process by which autoimmune T cells or activated T cells which persist following the successful clearance of an infection are triggered to undergo apoptosis[262,263,264]. Studies by Brunner et al., as well as other investigators revealing that soluble Fas can block ACID in several T cell lines lends support to this hypothesis by demonstrating that Fas is directly involved in removing potentially harmful T cells from the circulation[265,266,267].

In addition to its role in the regulation of immune system homeostasis, Fas-mediated apoptosis provides T- and natural killer (NK) cells with an alternative mechanism to the perforin lytic pathway for the induction of cytotoxicity in target cells[268,269,270]. Recent evidence suggests that the Fas system may play a key role in eliminating cancer cells[271,272], a hypothesis which is supported by data suggesting that (a) impairment of Fas receptor function can affect and modulate the process of tumor formation[273] and (b) soluble forms of Fas that may interfere with apoptotic signaling occur in patients suffering from various forms of lymphoid neoplasms[274].

Although the importance of soluble Fas as a factor which can acts physiologically to

limit apoptosis induced by FasL is becoming clear, in some cases, the source of soluble Fas is less evident. For example, Knipping et al., reported that soluble Fas harvested from condition medium of B-lymphoblastoid cells migrates to the same position as the membrane-bound form of Fas when analyzed by SDS-PAGE. Furthermore, since the amount of soluble Fas detected remained unchanged when the cells were cultured in the presence of conventional protease inhibitors, these authors concluded that soluble Fas was not generated by simple proteolytic cleavage from the cell surface, but rather that soluble Fas was directly secreted into the medium, presumably because it lacks a 19 amino acid transmembrane sequence; such a small deletion would not be resolved on SDS-PAGE[274,323] This conclusion, however, draws heavily from an earlier finding by Cheng et al., who described a soluble Fas molecule lacking the transmembrane domain because of a deletion in an exon encoding this region[275]. Regardless, an alterative explanation is possible: given that the methodology employed by Knipping et al., to isolate soluble Fas (i.e., centrifuging the condition medium at 100 000 x g and keeping the soluble Fas-containing pellet) is very similar to methods used to harvest vesicles[159,161,276], it is entirely conceivable that the *soluble* Fas described by these investigators is in fact, membrane-bound Fas released on shed vesicles. This hypothesis may explain the identical molecular masses of soluble and membrane-bound Fas reported by Knipping et al., and is supported by our own data indicating that Fas antigen is present on plasma membrane-derived vesicles released from the surface of colon adenocarcinoma cells.

Materials And Methods

Reagents and Chemical Suppliers:

2-amino-2-hydroxymethyl-1, 3-propanediol (Tris base), concentrated hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium hydroxide (KOH), sodium chloride (NaCl), sucrose, glycerol, Triton X-100, N-octyl-β-D-glycopyranoside, acetic acid, O,O'-Bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetra acetic acid (EGTA), ethylenediamine-tetra acetic acid (EDTA), cycloheximide, ammonium molybdate, chloroform, phenol, methanol, ethanol, cardolipin, acetic acid, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, and sodium citrate were purchased from Sigma Chemical Company (St. Louis, Mo.). Sodium dodecyl sulphate (SDS), bromophenol blue, agarose, acrylamide, bis-acrylamide and tetramethylethylene diamine (TEMED), Tween-20, and protein molecular markers were supplied by BioRad Laboratories (Mississauga, Ont.). Proteinase K, N-glyconase, ribonuclease A (RNAase A), adenosine triphosphate (ATP) phenylmethylsulfonyl fluoride (PMSF), leupeptin, propidium iodide and ethidium bromide and Hepes salt were obtained from Boehringer-Mannheim (Laval, Que.). Iodo-Beads, protein G beads, bicinchoninic acid (BCA) micro protein assay, goat IgG antibody and horseradish peroxidase were from Pierce (Rockford, IL.). [Methyl-^{3}H] choline chloride, ^{32}[γ-P]ATP, ^{125}I, ^{35}S-methionine, nitrocellulose membrane (0.2 micron), chemiluminescence substrate (ECL) were purchased from Amersham Life Science (Oakville, Ont.). Liquid scintillation cocktail (CytoScint) was from Dupont (Kingston, Ont.). Murine monoclonal anti-human Fas IgM antibody, CH.11, was obtained from Kamiya Biomedical Company (Thousand Oaks, CA.). Murine IgM antibody and *sn*-1,2-Diacylglycerol kinase were from

Calbiochem Corp. (La Jolla, CA.). Goat human anti-Fas antibody was was provided by Dr L. Owen-Schaub (University of Texas, MD Anderson Cancer Center, Houston, Texas). Murine polyclonal anti-Fas antibody (BMS 140) and rabbit polyclonal anti-FasL antibody (Ab-1) were purchased from Cederlane Laboratories (Hornby, Ont.). Rabbit anti-mouse IgG (H + L) was supplied by Jackson Immuno Research Laboratories (Mississauga, Ont.). Magnesium chloride ($MgCl_2$), sodium fluoride (NaF), glass slides, thin layer chromatography (TLC) silica gel plates and coverslips were from Fisher Scientific Company (Montreal, Que.). Anti-flt3/flk2 antibody and flt3/flk2 plasmid DNA were obtained from Immunex Corporation (Seattle, WA).

Instrumentation:

Flow Cytometric analysis was performed using a flow analyser model XL (Coulter). Vesicles were counted or sorted on a FACS VANTAGE sorter (Becton-Dickinson). Radioactive counts were obtained using a model 1219 Rackbeta liquid scintillation counter from Fisher Scientific Company. Electron micrographs were obatined using a Philips CM10 transmission electron microscope. Fluorescence microscopy was performed on a Optiphot-2 microscope from Nikkon (Mississauga, Ont.) and photographs obtained using a Nikkon camera (model FX-35DX). Absorbancies for colorimetric assays (MTT and protein) were read on a MR5000 microplate reader from Dynatech (Chantilly, VA). UV-B lamp (Spectroline series E) used in irradiation studies was obtained from Fisher Scientific Company. High speed centrifugations were performed using a model L7-55 utracentrifuge from Beckman (Montreal, PQ) and lowspeed centrifugations were performed using a mode J-6M centrifuge, also from Beckman.

Tissue Cultures:

Human colorectal adenocarcinoma cell lines, CX-1 and MIP-101, were a generous gift from Dr P. Thomas (Deaconess Hospital, Harvard Medical School, Boston, MA.). The Human bone marrow stromal cell line was a gift from Dr B. Torok-Strob (Hutchinson Cancer Research Center, Seattle WA.). The Chinese hamster ovary cell line, CHO-K1, and the human T cell line, HuT 78, were obtained from American Type Culture Collection (Rockville, MD.). Roller bottles, T225 tissue culture flasks, plastic tubes (11.5 x 75 mm), plastic centrifuge tubes (50 m), polystyrene dishes (100 mm diameter), nylon mesh filters (30 microns) and 96 well microtiter plates were obtained from Falcon; Becton-Dickinson (Franklin Lakes, NJ.). T75 tissue culture flasks were purchased from Costar Corporation (Cambridge, MA.). Phosphate buffered saline (PBS), RPMI-1640 (RPMI) and Ham's F12 (F12) powder, geneticin (G418), penicillin and streptomycin (P/S) were obtained from Life Technologies; Gibco BRL (Grand Island, NY.). Fetal calf serum (FCS) and trypan blue stain (0.4%) were purchased from BioWhittaker Inc. (Walkersville, MD.).

Human colorectal adenocarcinoma cell lines, CX-1 and MIP-101, human T cell line, HuT 78, and human bone marrow stromal cell line, HS5, were cultured in T225 tissue culture flasks. Cells were maintained in RPMI-1640 (RPMI) medium supplemented with 10% fetal calf serum and containing 100 units/ml of penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2, at 37°C. CHO-K1 cells were cultured in F10 medium, and maintained as just described. Prior to harvesting vesicles, cells were habituated to proliferate under low serum conditions as follows: Adherent cells (MIP-101, CX-1, HS-5 and CHO-K1) in confluent flasks were washed 3 times with phosphate buffer

saline, pH 7.2 (PBS) and gently detached with 15 ml PBS containing 15 mM sodium citrate, pH 7.2. Following centrifugation at 800 x g for 5 min, the citrate solution was aspirated and the cells were resuspended in 20 ml RPMI (or F10 medium for CHO-K1 cells) containing 8% FCS and P/S. Two ml of the cell suspension were introduced into T225 culture flasks containing 50 ml of RPMI supplemented with 8% FCS and P/S. Cells were allowed to reach confluency and passaged once again, in a serum concentration that was further reduced by 2%. This process was repeated until the cells were maintained in media containing 2% FCS and P/S. When cells were 60 -70% confluent, media was aspirated, cells were washed 3 times with PBS and 200 ml of fresh, serum-free RPMI were added to each flask. Flasks were returned into the incubator and cells allowed to grow for 16 - 18 hr. Suspension cells (HuT 78) were harvested by centrifugation at 800 x g for 5 min, washed 3 times in PBS and treated as described for adherent cells. Typically, greater than 95% of cells were capable of excluding trypan blue.

UV-B Irradiation:

CHO-K1 cells were washed 3 times with 30 ml phosphate buffered saline (PBS) following aspiration of the media and detached with 25 ml PBS-citrate. Cells were harvested by centrifugation (5 min at 700 x g) and resuspended in 5 ml PBS. The suspension was filtered through a nylon filter (45 micron) to remove clumps. Enough PBS was added to make a suspension of 5×10^6 cells/ml. Aliquots of 2 ml were placed in polystyrene dishes (100 mm diameter) and subjected to a single dose of radiation obtained from a UV-B lamp. Energies of 300 and 600 Joules/m^2 were delivered by adjusting the lamp 3.5 and 3.0 inches above the samples and irradiating cells for 100 and 95 sec, respectively. Energies of 1200, 1600 and

2800 Joules/m^2 were delivered by positioning the lamp 1 inch above the samples and irradiating the cells for 100, 130 and 215 sec, respectively. Control samples were treated in an identical fashion, except that they were not subjected to irradiation. Following irradiation, treated and untreated cells were incubated with 10 ml Ham's F-12 medium containing no FCS. At indicated times cells were harvested as follows: (1) media from the dish was placed in a 50 ml centrifuge tube and the attached cells were dislodged from the dish with 5 ml PBS-citrate. (2) the cell suspension was combined with the medium. This step was repeated twice more. (3) cells were centrifuged for 5 min at 700 x g and resuspended in 5 ml PBS. Viability was determined by trypan blue exclusion.

Cell surface labeling:

Eighteen hr before cell surface labelling, the medium in tissue culture flasks was aspirated and the cells washed 3 times with 30 ml PBS. Cells were incubated overnight in 100 ml serum-free RPMI media. On the day of the experiment, medium was aspirated and the cells were washed 3 times with 30 ml PBS and detached with 25 ml PBS-citrate solution. Cells were harvested by centrifugation, resuspended in 2 ml PBS and filtered through a 30 μm nylon mesh to remove clumps. The volume was adjusted to 3.5 ml with PBS and cells were surface labeled by combining two Iodo-Beads and 2 mCi ^{125}I to 1 x 10^7 cells. The cells were gently mixed by slowly inverting the 5 ml tube for 1 min, and the reaction was quenched by removing the beads. The cells were pelleted by centrifugation for 5 min at 700 x g and washed 3 times with 5 ml PBS. Radiolabeled cells were then cultured in serum-free RPMI under the same conditions described above.

Kinetic Studies And Vesicle Preparation:

Serum-free conditioned medium was decanted from flasks and centrifuged at 2000 x g for 10 min to remove cells and debris. The supernatant was subjected to ultracentrifugation at 100,000 x g for 12 hr at 8°C. Supernatant was discarded and the vesicles were washed twice by resuspending the pellet in PBS, followed by ultracentrifugation at 100,000 x g for 12 hr at 8°C. Vesicles were used immediately or stored at -80°C until needed. Vesicles collected from ^{125}I-labeled cells were resuspended in 5 ml scintillation cocktail and the amount of radioactivity (cpm) was determined by scintillation counting spectrometry.

Plasma Membrane Preparation:

With the exception of CHO cell, cell cultures were grown in T225 culture flasks and incubated as described above. CHO cells were cultivated in roller bottles. Unless otherwise stated, 2 x 10^9 cells were used for membrane preparations. Adherent cells were detached with 25 ml PBS-citrate solution (10 ml for cells grown in T225 flasks) and harvested by centrifugation at 700 x g for 5 min. The pellet was resuspended in 5 ml PBS, 833 ul 90% (w/v) glycerol dissolved in PBS (PBS-glycerol) were added and the mixture incubated at 37°C for 5 min. Two more aliquots (833 ul) PBS-glycerol were added and the cell suspension was incubated at 37 °C for 5 min after each addition. Subsequently, the suspension was centrifuged (700 x g, 10 min), the pellet resuspended with 9 volumes (with respect to the pellet) 5 mM Tris-HCl (pH 10) containing 2 mM $MgCl_2$, 10 ng/ml leupeptin and 35 mg/ml PMSF, and allowed to stand at room temperature for 2 min. From this point on, all steps were performed at 4°C. Cells were disrupted using a Dounce homogeniser (20 strokes) and then by gently aspirating the suspension through a 25 gauge needle (10 times).

The lysate was centrifuged (700 x g, 10 min) and the nuclei-rich pellet discarded. The supernatant was centrifuged at 3000 x g for 10 min and the pellet discarded. Plasma membrane suspended in the supernatant were harvested by centrifugation at 37 000 x g for 45 min, the pellet resuspended in 3 ml 5 mM Tris-HCl (pH 7.4) containing 20% (w/v) sucrose and the suspension gently aspirated through a 25 gauge needle. The mixture was layered over a sucrose-step gradient composed of 50, 40 and 30% (upper-most layer) sucrose in 5 mM Tris-HCl, pH 7.4, that was established in a 12.5 ml ultracentrifuge tube. The preparation was centrifuged at 200 000 x g for 16 hr. The plasma membrane band which formed at the 30-40% interface was transferred to a new 12.5 ml ultracentrifuge tube, diluted with 10 ml PBS and subjected to ultracentrifugation at 200 000 x g for 16 hr. The plasma membrane pellet was used immediately or stored at -80 °C.

Propidium Iodide DNA Staining:

CHO cells were detached with PBS-citrate, washed once with PBS, resuspended in 2 ml PBS-citrate and incubated for 5 min at 37°C. Cells were collected by centrifugation at 700 x g for 5 min, washed once in 5 ml PBS-FCS and the cells harvested by centrifugation. The cell pellet was resuspended in 0.5 ml PBS and fixed by drop-wise addition of 5 ml 90% ethanol (-20 °C) while gently vortexing the sample in order to prevent clumping, and then incubated overnight at 4°C. Subsequently, the cells were centrifuged at 800 x g for 5 min and washed once with PBS-FCS and pelleted by centrifugation. The pellet was resuspended in 1 ml freshly prepared propidium iodide/RNAase solution (20 µg/ml propidium iodide, 250 µg/ml RNAase A (boiled for 15 min), 1.5 mM sodium citrate, 375 µM NaCl in 250 µM Tris-HCl, pH 7.0) and incubated for 30 min at 37°C.

Lipid Analysis:

Sphingomyelin was quantified in shed vesicles and plasma membranes isolated from CHO cells previously cultured and maintained for 5 days in F12 medium containing 2% FCS and 0.25 mCi/ml [methyl-^{3}H]choline, as described by Dressler et al., (1992)[277]. Tritium-labeled cells were washed 3 times with PBS, incubated for 18 hr in serum-free medium and shed vesicles collected as described above. In studies where plasma membranes were required, labeled cells were washed 3 times with PBS, harvested, and their plasma membranes purified as described above. After irradiation at 4°C, extracellular vesicles and plasma membranes were incubated at 37°Cin a mixture of 3 mM ATP, homogenization buffer (50mM NaF, 5 mM EGTA and 25 mM Hepes, pH 7.4), distiled water and sphingomyelinase assay buffer (50 mM Hepes, pH 7.4 and 20 mM $MgCl_2$) (30:30:90:300, v/v). At indicated times, lipids in plasma membranes or shed vesicles were extracted with chloroform/methanol/hydrochloric acid (1N) (500:500:5 v/v). The organic phase was dried under nitrogen and glycerophospholipids removed by treating the lipid mixture with 200 ul 1M KOH in methanol for 1 hr at 37 °C. Lipids were separated on thin layer chromatography)using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50:30:8:4, v/v) as solvent, identified with iodine vapour staining and radioactivity present in the sphingomyelin bands quantified by liquid scintillation spectroscopy.

Ceramide in shed vesicles and purified plasma membranes was measured by the diacylglycerol kinase assay as ^{32}P incorporated in ceramide following its phosphorylation to generate ceramide-1-phosphate, a reaction catalysed by diacylglycerol kinase. Cells were treated as described for sphingomyelin quantization, except that labeling with tritiated

choline was omitted. Following UV-B treatment, shed vesicles and purified plasma membranes were extracted with chloroform/methanol/hydrochloric acid (1N) and the organic phase transferred to 5ml tube. Twenty microliters of a solution containing 7.5% n-octyl-β-D-glcopranoside, 5 mM cardiolipin and 1 mM DTPA were added to the organic phase extract. To this mixture, 0.1 mg of diacylglycerol kinase contained in 40 µl diacylglycerol kinase enzyme buffer (20 mM Tris-HCl, 10 mMdithiothreitol, 1.5 M, 250 mM sucrose and 15% glycerol, pH 7.4) were added and the enzymatic reaction initiated following the addition of 20 µl 10 mM γ-[^{32}P]ATP in enzyme buffer. The reaction was allowed to proceed for 30 min at room temperature and then, stopped by addition of 1 ml $CHCl_3/CH_3OH/HCl$ (100:100:1, v/v), 170 µl saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM Hepes, pH 7.2) and 30 µl 100 mM EDTA. The organic phase was collected and dried under nitrogen. To the lipids were dissolved in 15 ul $CHCl_3/CH_3OH/CH_3COOH$ (65:15:5, v/v) and ceramide-1-phosphate separated by thin layer chromatography. The radioactive ceramide-1-phosphate band was detected by autoradiography and the amount of ^{32}P incorporated in the lipid was determined liquid scintillation spectrometry.

MTT Viability Assay:

Cell viability was correlated to the capacity of cells to reduce 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to formazan, as described by the manufacturer of the MTT assay. Briefly, 2 x 10^5 cells were cultured in wells of a 96-well microtiter plate for 24 hr under the conditions described above. Following the incubation period, media was removed and replaced with 200 ul fresh RPMI media containing 10% FCS, cyclohexamide (10 ug/ml), and 100 ng isotype-matched antibody (IgM) as a control or 100 ng murine monoclonal anti-

human Fas antibody, CH.11. To appropriate wells were added 20 µl of vesicles (prepared from CX-1 or MIP-101 cells that were resuspended in PBS) containing 40 µg total protein. Cells were incubated for 24 hr, after which MTT reagent was added and the cells were incubated for an additional 4 hr. Levels of formazan were quantified with a microplate reader at 570 nm.

Relative resistance of CX-1 and MIP-101 cell lines to anti-Fas-induced cell death was determined in a similar fashion with the exception that RPMI media was not replaced following the initial 24 hr incubation period; all reagents (cyclohexamide and antibodies) were added to the conditioned media, as described above.

Fas Biosynthesis:

Biosynthesis of Fas was measured by determining the amount of ^{35}S-methionine incorporated into the newly synthesized protein. CX-1 and MIP-101 cells maintained in RPMI supplemented with 10% FCS medium were gently detached by treatment with 15 mM citrate-PBS solution, and harvested by centrifugation at 800 x g for 5 min. Cells (1.5×10^7) were washed twice with PBS and resuspended in methionine-free RPMI-1640 media supplemented with 10% FCS at a density of 5×10^5 cells/ml, and 300 µCi ^{35}S-methionine was added. Cells were incubated for 2 or 16 hr at 37°C in a humidified atmosphere with 5% CO_2. Trypan blue staining revealed that at the time of harvest, greater than 95% of the cells excluded dye.

Immunoprecipitation:

^{35}S-Methionine-labeled CX-1 and MIP-101 cells (1×10^7) were collected by centrifugation at 800 x g for 5 min. Pellets were dissolved in 1 ml 50 mM Tris-HCl buffer,

pH 8.0, containing 0.5% Triton X-100, 10 mM EDTA and 150 mM NaCl. Subsequently, suspensions were mixed by inversion while incubating at 4°C for 30 min. Following solubilization, lysates were dialyzed against 0.05% Triton X-100 in Tris-HCl buffer at 4°C for 16 hr. The radiolabeled samples were cleared with goat IgG immobilized on protein G beads and Fas was immunoprecipitated by incubating the mixture with goat anti-Fas bound to protein G beads at 4°C for 2 hr. Beads were washed with 0.05% Triton X-100 in Tris-HCl buffer 3 times, and resuspended in 75 µl 2 X SDS PAGE sample buffer (0.25 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.1% bromophenol blue). Samples were placed for 5 min in a water bath at 100°C, centrifuged in a minifuge for 10 sec, and the pelleted beads were discarded. A 15 µl aliquot of each sample was retained to determine the amount (cpm) of ^{35}S-methionine incorporated in immunoprecipitated Fas. The remainder of each sample was electrophoresed in a 7.5% polyacrylamide gel, and the bands were visualized by autoradiography.

Protein Assay:

Protein levels were determined using micro BCA assay, according to the protocol suggested by the manufacturer.

Electron Microscopy:

Exfoliated vesicle samples were placed on a formvar-carbon coated 200 mesh copper grid that was glow discharged just before application. Samples were allowed to adhere for 1 min, then wicked off with filter paper and allowed to air dry. The grids were blocked with 2% BSA in PBS for 30 min, then incubated for 1 hr with mouse anti-Fas IgM (10 ul/ml). After 3 rinses in PBS and another block for 10 min, the grids were incubated for 2.5 hr at 4

°C on drops of goat anti-mouse IgM labeled with 10 nm gold particles. The grids were rinsed 3 times in PBS, then given 3 rinses with distilled water, stained for 1 min with 2% ammonium molybdate, dried, and examined and photographed in a Philips CM10 transmission electron microscope at 60 kV.

Agarose Gel Electrophoresis of DNA Samples:

Irradiated (600 Joules/m^2 CHO cels (1×10^7) were incubated for 2, 4, 6, 8, 10, and 18 hr in serum-free F12 medium. Cells were harvested as described above and washed 3 times with PBS. Subsequently, cells were centrifuged at 800 x g and the pellet treated with 0.5 ml lysis buffer consisting of 100 mM NaCl, 1mM EDTA, 1% SDS and 100 µg protinase K dissolved in10 mM Tris-HCl, pH 7.8. The cell suspension was incubated overnight at 37 °C, 2 µl RNAase (10 mg/ml) was added and the mixture incubated for another hour at 37 °C. DNA was precipitated by adding 1.5 ml 1 M NaCl in 80% ethanol (-20 °C) followed by overnight incubation at -20 °C. Precipitated DNA was transferred to a new tube and dissolved in 200 µl Tris-borate EDTA buffer (TBE) consisting of 90 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0) and the concentration determined by UV spectroscopy (λ = 260 nm).

Western Blot Analysis:

Expression of Fas and FasL was determined by Western blot analysis. Solubilized plasma membranes or plasma membrane-derived vesicles were immunoprecipitated and electrophoresed in SDS-7.5% polyacrylamide. Resolved protein bands were transferred to nitrocellulose, blocked overnight with Tris-buffered saline (20 mM Tris buffer, 137 mM NaCl , pH 8.0) supplemented with 0.1% Tween-20 (TBS-T), and incubated for 1 hr at room temperature with either murine polyclonal anti-Fas antibody or rabbit polyclonal anti-FasL

antibody diluted 1000-fold. The nitrocellulose membrane was washed three times with 50 ml TBS-T. Lanes treated with murine anti-Fas antibody were incubated for 1 hr with rabbit anti-mouse IgG (H + L) diluted 1000 -fold in TBS-T and then washed three times with TBS-T. Subsequently, all lanes were treated for 30 min with horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 5000-fold in TBS-T. Blots were developed with chemiluminescence substrate.

Fas antigen was deglycosylated as folllows: to 200 µl of plasma membranes suspended in PBS was added sufficient SDS to give a final concentration of 0>15%. The sample was boiled for 5 min and the pH adjusted to 7.8. Following the addition of three units N-glyconase to the aliquot, the ample was incubated at 37 °C for 18 hr. Deglycosylated proteins were solubilized, immunoprecipitated and subjected to Western blot analysis as described above.

Plasma membranes and plasma membrane-derived vesicles from HS-5 and CHO-K1 cells transfected with cDNA encoding for flt-3/flk-2 ligand were solubilized as described under the *immunoprecipitation section.* The samples were then cleared with either murine IgG antibody (for plasma membranes and plasma membrane-derived vesicle extracts from HS-5 cells) or with rabbit IgG antibody (for plasma membranes and plasma membrane-derived vesicle extracts from CHO-K1 cells) bound to protein G beads. Subsequently, plasma membrane and vesicle extracts were immunoprecipitated with murine anti-M-CSF (HS-5 cells) or with rabbit anti-flt3/flk2 ligand antibody. The presence of M-CSF or flt3/flk2 ligand in immunoprecipitates were detected by Western blot analysis as described above.

Immunofluorecent Microscopy and Flow Cytometry:

MIP-101 and CX-1 cells were cultured in T75 flasks as described above. Cells were washed three times with PBS and detached with 10 ml PBS-citrate solution. The cell suspension was centrifuged at 300 x g for 5 min, and the pellet was then resuspended in PBS containing 1% FCS (PBS-FCS) at a concentration of 2 x 10^6 cells/ml. One hundred microliters (2 x 10^5 cells) of the cells suspension were transferred to 11.5 x 75 mm plastic tubes and 200 ng of isotype-matched antibody (IgM) as a control or 200 ng murine monoclonal anti-human Fas antibody (CH.11) were added. The cells were mixed gently and placed on ice for 20 min. Following the incubation period, the cells were washed twice with 2 ml PBS-FCS (centrifuged at 300 x g for 5 min) and 100 µl (50 µg/ml) of goat anti-mouse IgG/IgM, (H + L)-fluorescein conjugated antibody were added. The cells were mixed gently and the tubes placed on ice for 20 min. The cells were washed twice with PBS-FCS and harvested by centrifugation at 300 x g for 5 min. The cell pellet was resuspended in 50 µl mounting fuid (1 part PBS and 9 parts glycerol), mounted on slides under coverslips and sealed with nail varnish. Cells were examined under fluorescence illumination using an Optiphot-2 microscope equipped with a 40 x magnification objective. Photographs were taken using a Nikkon camera.

Vesicles were harvested from serum-free medium conditioned by CX-1 cells or MIP-101 cells and enumerated on a FACS VANTAGE sorter. Briefly, 5 x 10^6 vesicles were resuspended in 2 ml PBS-FCS to which 100 ng mouse anti-human Fas IgM (CH.11) antibody were added. Samples were incubated at 4 °C, 30 min, and vesicles were washed as follows: The sample volume was increased to 30 ml with PBS-FCS and the vesicles were pelleted by

centrifugation at 100 000 x g at 4°C for 30 min in the presence of rabbit FITC-conjugated anti-mouse IgM (20 μg/ml). Vesicles were washed, resuspended and analyzed by flow cytometry.

Calcium Phosphate Transfection:

Stable expression of flt3/flk2 lignad in CHO cells was established by cotransfecting cells using the calcium phosphate precipitation method as follows: Cells (1×10^5) where split and seeded into 10 cm tissue culture plates the day before the transfection. On the day of the transfection, the cells were washed once in PBS and feed with 9 ml Ham's F12 medium supplemented with 10% FCS and P/S four hours prior to addition of precipitated DNA. To form the DNA precipitate, 15 μg pDC414 (flt3/flt2 plasmid) and 2 ug pSVneo (G418 resistance plasmid) were added to 400 μl sterile water; the volume was then adjusted to 450 μl with water. Fifty microlitres 2.5 M $CaCl_2$ were added to the plasmid DNA solution. The DNA/$CaCl_2$ solution was added dropwise, using a Pasteur pipet, to 500 μl 2x HeBS placed in a 15 ml conical tube. To ensure formation of a fine calcium phosphate precipitate, a mechanical pipettor attached to a 1 ml plugged pipet was used to bubble the mixture while adding the DNA/$CaCl_2$ solution. The mixture was immediately vortexed and allowed to incubate at room temperature for 20 min. Using a Pasteur pipet, the precipitate was evenly distributed over the CHO cells and the 10 ml culture dish was gently agitated to mix the precipitate and medium. The cells were incubated for an additional 6 hr as described above, the medium was removed, the cells washed once with PBS and feed with 9 ml fresh medium. The cells were allowed to grow for 48 hr. At this point, the medium was removed, the cells were detached (2 ml PBS/citrate) and plated in selective medium containing 300 μg/ml G418.

Chapter 3 Results

Section 3.1: Plasma membrane as a sensitive target in UV-B-induced cells damage

It was previously observed that UV-B irradiation induces changes the ultrastructure of the lymphocyte plasma membrane, resulting in the loss of villi [278]. In order to establish a time-course of alterations in the plasma membrane relative to those in the nucleus, CHO were exposed to a single dose of UV-B radiation ranging from 0 - 2800 Joules/m^2. As figure 7 shows, energies greater then 600 Joules/m^2 resulted in a dose-dependent decrease in cell viability following UV-B irradiation. Sub-lethal doses of 300 and 600 Joules/m^2 resulted in no significant difference in cell viability as compared to unirradiated cells (control) after 18 hr. Comparatively, 50, 37 and 25% of CHO cells treated with doses of 1200, 1600 and 2800 Joules/m^2, respectively, were able to exclude trypan blue dye after 18 hr.

To determine whether UV-B radiation induces DNA fragmentation in CHO cells, DNA of cells extracted at various times post-irradiation (600 Joules/m^2) was subjected to agarose gel electrophoresis (figure 8). Our results indicate that UV-B irradiation induced intranucleosomal DNA fragmentation typically observed in cells undergoing apoptosis. Following electrophoresis, a DNA ladder pattern was apparent after 4 hr following UV-B irradiation and the intensity of the bands increased over 18 hr, suggesting an increase in DNA cleavage over time following irradiation. Consistent with these data are the results obtained from flow cytometric analysis of CHO cells stained with propidium iodide posts UV-B treatment. As figures 9 and 10 show, there is an increase in the fraction of cells with a DNA contents less then 2N with time following irradiation with 600 Joules/m^2 UV-B. Unirradiated control samples show no significant increase in the fraction of cells with decreased DNA content after 18 hr. At this time, the fraction of CHO cells with DNA < 2N

Figure 7: Effects of UV-B on viability of CHO cells. Shown are viabilities over time following radiation treatment with 0 (filled squares), 300 (filled triangles), 600 (open triangles), 1200 (diamonds), 1600 (circles), or 2800 (open squares) Joules/m^2 UV-B. Note a concentration-dependent reduction in cell viability over time. Shown are means ± SEM of three experiments.

Figure 8: Agarose gel electrophoresis of DNA extracted from CHO cells treated with UV-B (600 Joules/m^2). Note the increasing intensity of internucleosomal DNA fragment bands over time. Lanes 2 - 18 represent the time (hour) at which DNA was extracted from CHO cell following irradiation. A one hundred basepair ladder (Lad) was used for size reference.

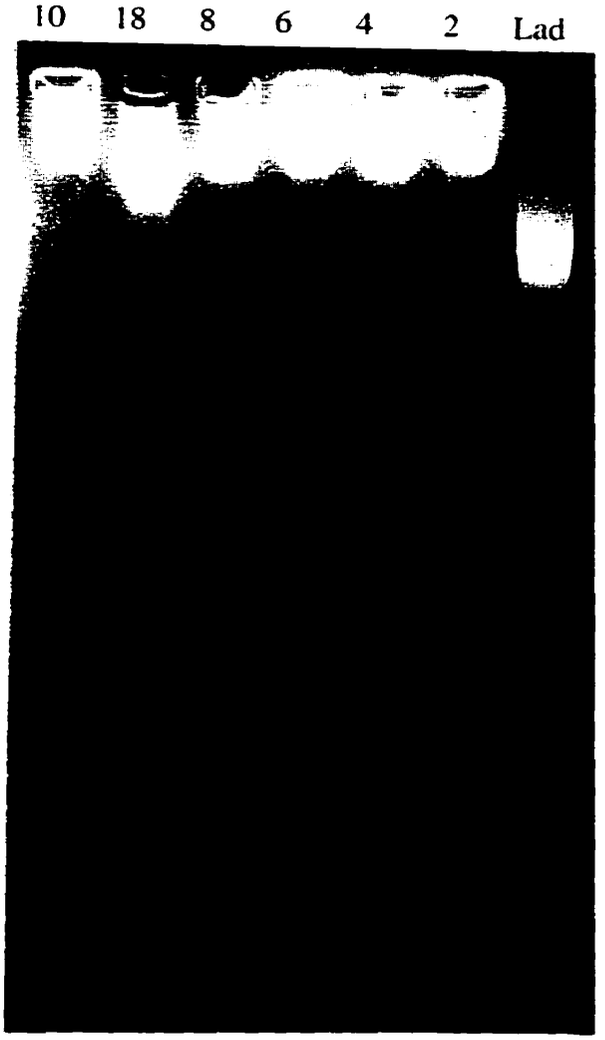

10
18
8
6
4
2
Lad

Figure 9: UV-B induces DNA fragmentation. CHO cells irradiated with 600 Joules/m^2 UV-B and stained with propidium iodide were analyzed flow cytometry, as described in *materials and methods*. Note that the proportion of cells with DNA content less then 2N for irradiated cells (solid bars) increases over an 18 hr period post-irradiation, while that for unirradiated cells (open bars) remains constant and significantly lower at all time points, relative to treated cells. Shown are mean ± SEM values of three separate experiments.

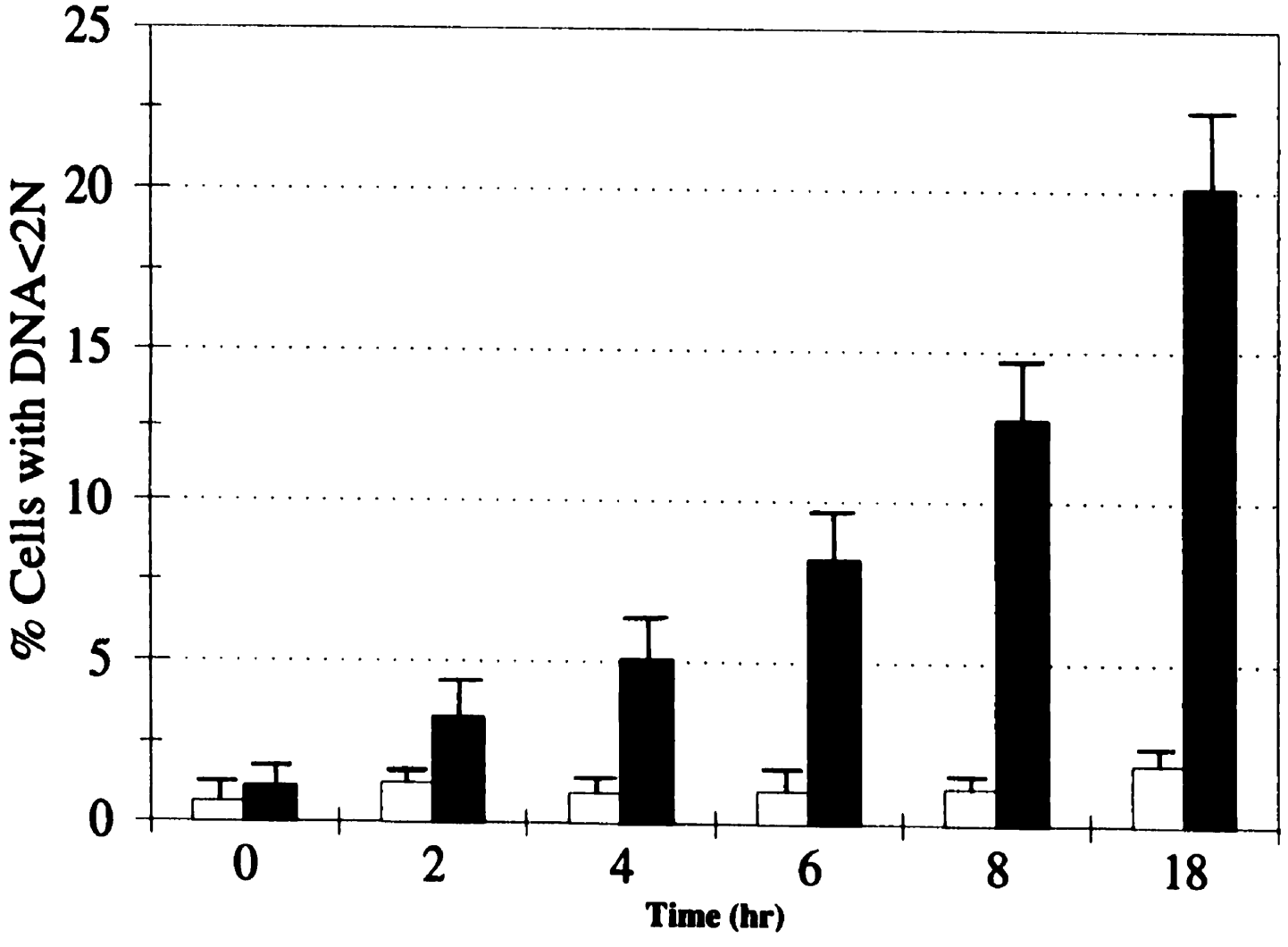
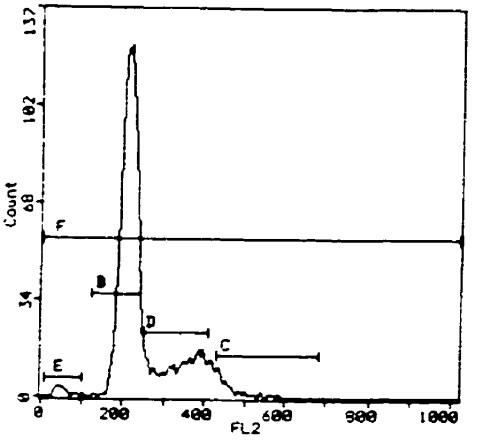

25
20
15
10
5
0
% Cells with DNA<2N
0
2
4
6
8
18
Time (hr)

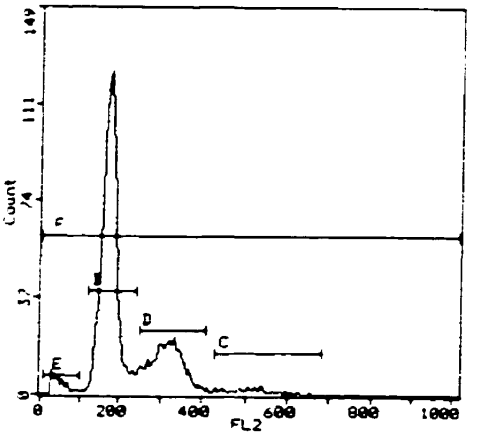
Figure 10: Flow cytometric analysis for unirradiated and irradiated (600 Joules/m^2) CHO cells stained with propidium iodide. Shown are histograms typically obtained for control (left) and irradiated (right) cells at 4, 8, and 18 hr post-treatment. The hypodiploid peak (E) corresponds to the apoptotic cell population. Note the increase in the height of this peak over time.

Control

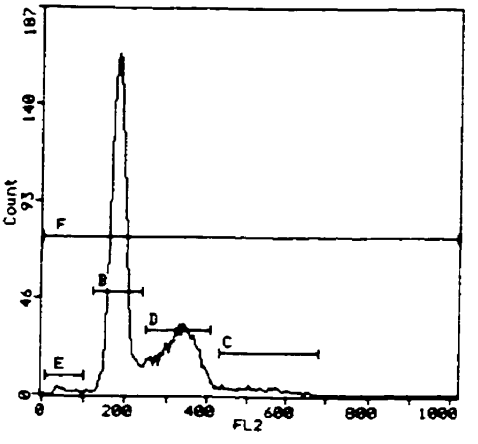
600 J/m^2 UV-B

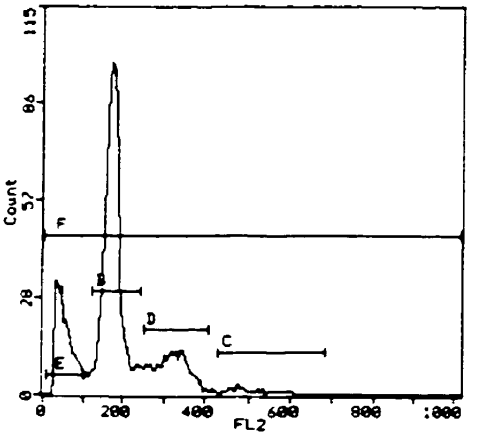
4 hours



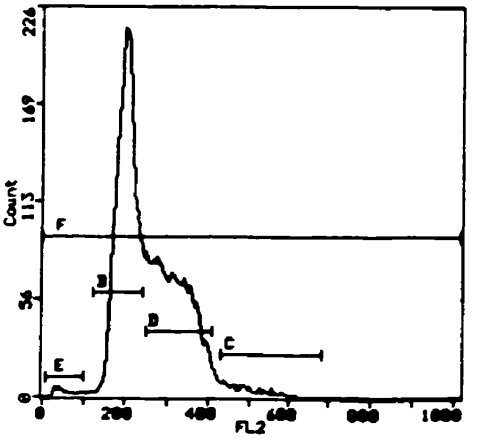


8 hours





16 hours



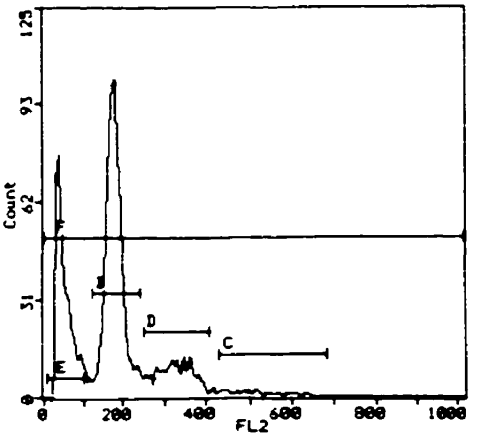
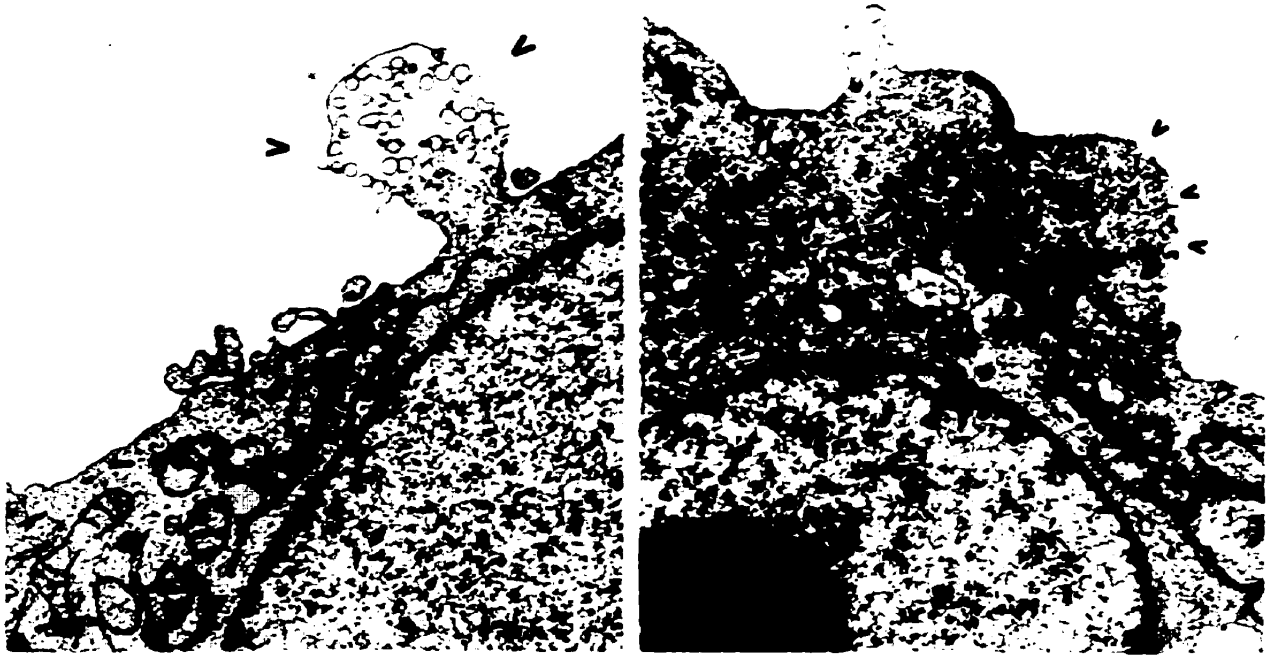
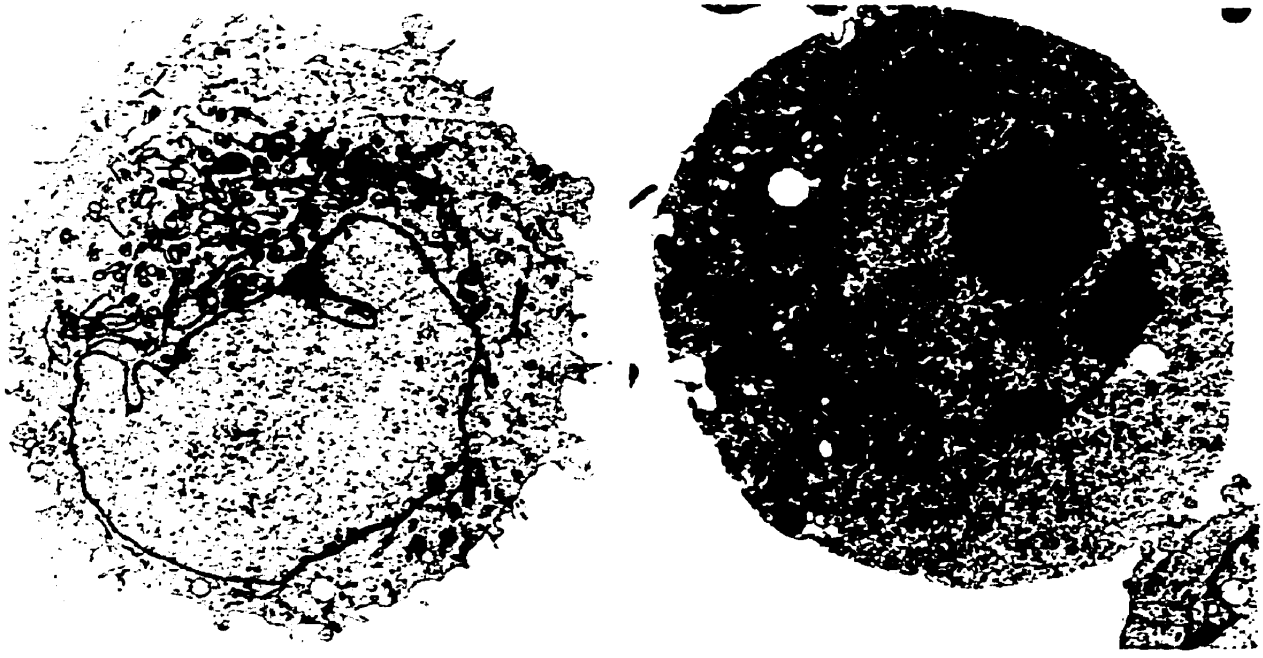


Figure 11: Effects of UV-B (600 Joules/m^2) on cell ultrastructure. CHO cells were irradiated and fixed for electron microscopy, as described in the *materials and methods* section. Note that small protrusions are observed after 1 hr (upper left panel) and by 6 hours (lower left panel), numerous areas of protrusions are evident. Protrusions appear to contain membrane vesicles, and are devoid of cytosol or cytosolic components. Three hours (upper right panel) post-irradiation, fenestrations are observed on the plasma membrane. By 16 hr (lower right panel), the cell surface is smooth and morphological changes characteristic of cells undergoing apoptosis (i. e., swelling of the mitochondria and condesation of nuclear chromatin) are seen.



1 hour

3 hours



6 hours

16 hours

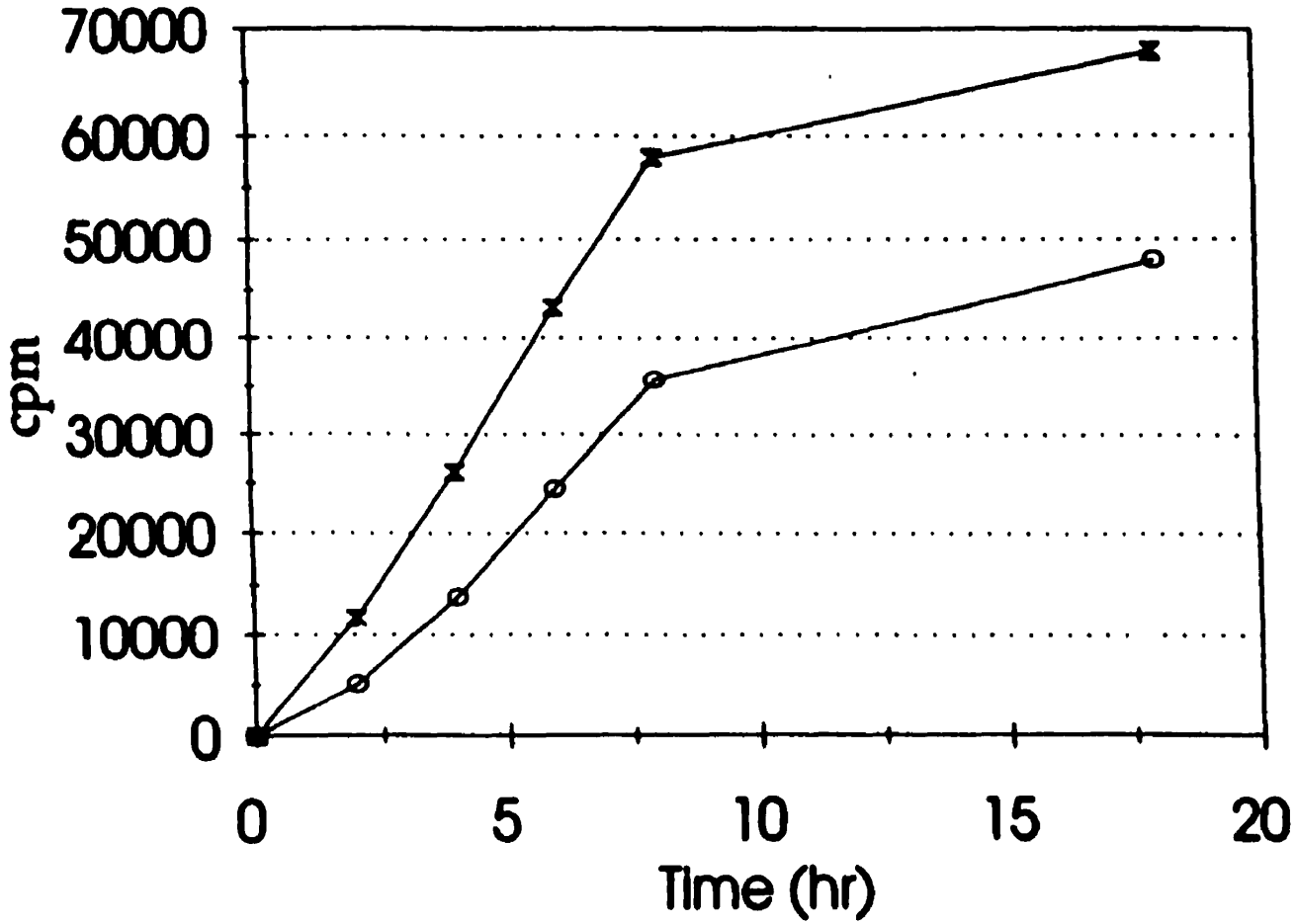
extend into the protrusions. Cytoplasmic components and the nucleus exhibited normal morphology at this time. As time elapses beyond 6 hr, changes typical for apoptosis became apparent. Among these, the formation of chromatin clusters as well as chromatin margination in the nucleus, and swelling of the mitochondria evident16 hr post-irradiation. At this time, 30 - 35% of the CHO population displayed these morphological alterations.

At this point, it is appropriate to mention that 1 hr following UV-B treatment, 85 - 90% of CHO cells failed to exhibit plasma membrane protrusions and/or fenestrations. However, the proportion of cells with radiation-induced plasma membrane alterations increased to 35% 6 hr following treatment. An increase in the proportion of cells with plasma membrane damage over time following low-dose gamma irradiation was also reported by Chandra and Stefani (1981)[86]. Additionally, these investigators point-out that cells which appear normal (those lacking protrusions and/or fenestrations) may exhibit plasma membrane damage in another plane of sectioning.

3.1.1 Shedding of extracellular vesicles is impaired following UV-B irradiation

Cell surface constituents (proteins, lipoproteins, sphingolipids and glycosaminoglycans) are continually and selectively released from the plasma membrane on extracellular vesicles via exfoliation. This phenomenon was been shown to be a directed process, occurring from distinct areas of the plasma membrane and depends on oxidative phosphorylation, transcription and post-translational modification[158, 281, 282]. To determine whether UV-B radiation-induced morphological alterations of the plasma membrane in CHO cells observed at early time points coincide with accelerated rates of vesiculation versus decreased shedding,

Figure 12: Cumulative shedding of extracellular vesicles is reduced by UV-B radiation. Release of plasma membrane-derived vesicles into serum-free medium conditioned by surface-^{125}I-labeled CHO cells was quantified by measuring the amount of radioactivity (cpm) in the pellet, following their harvest by highspeed centrifugation (see *material and methods*). Note that cells exposed to 600 Joules/m^2 UV-B (circles) exhibit decreased cumulative release of extracellular vesicles relative to control cells (hourglasses).


70000
60000
50000
40000
30000
20000
10000
0
cpm
0
5
10
15
20
Time (hr)

vesicles exfoliated from the surface of ^{125}I-labeled cells (see Materials and Methods) into serum-free medium were collected by isopycnic centrifugation and the radioactivity in the vesicle-enriched pellet was quantified by scintillation counting. As is shown in figure 12, relative to unirradiated (control) cells, the cumulative amount of radioactivity released from UV-B treated cells in association with extracellular vesicles is decreased at all time points. The greatest difference in rate of exfoliation (figure 13) is seen at early time points, beginning as early as 2 hr and persisting up to 6 hr. This finding complements the ultrastructural analysis (figure 11) which provides morphological evidence for the appearance of vesicles at the cell surface. Together, these results suggest that extracellular vesiculation is diminished following exposure to low-doses of UV-B radiation, resulting in *retention* of plasma membrane-derived vesicles at the cell surface. Ultimately, these vesicles appear to be incorporated into the plasma membrane as the cell surface becomes smooth in appearance.

Many studies have shown that ceramide acts as a second messenger to modulate apoptosis. Moreover, a report by Haimovitz-Friedman et al., (1994) provides strong evidence that X-rays induce the hydrolysis of sphingomyelin to generate ceramide. Our observation that UV-B radiation diminishes shedding (figures 12 and 13) and promotes retention of shed vesicles on the cell surface prompted us to investigate whether UV-B irradiation induces the generation of ceramide in extracellular vesicles derived for the cell surface of CHO cells. Plasma membranes and shed vesicles derived from CHO cells were exposed to 600 Joules/m^2 UV-B radiation and levels of sphingomyelin and ceramide were determined. Fifteen sec following treatment, the level of sphingomyelin in shed vesicles

Figure 13: Rate of shedding is reduced following exposure to UV-B. The surface of CHO cells was labeled with ^{125}I. Plasma membranes-derived extracelluar vesicles shed into serum-free medium conditioned by UV-B-treated and control (untreated) cells were harvested at indicated times of incubation. Rates of labeled membrane-protein released on shed vesicles were calculated as counts per minute (cpm) per hour. Note the marked reduction in rate of release at 2 hr from irradiated cells (circles), relative to untreated cells (hourglasses).

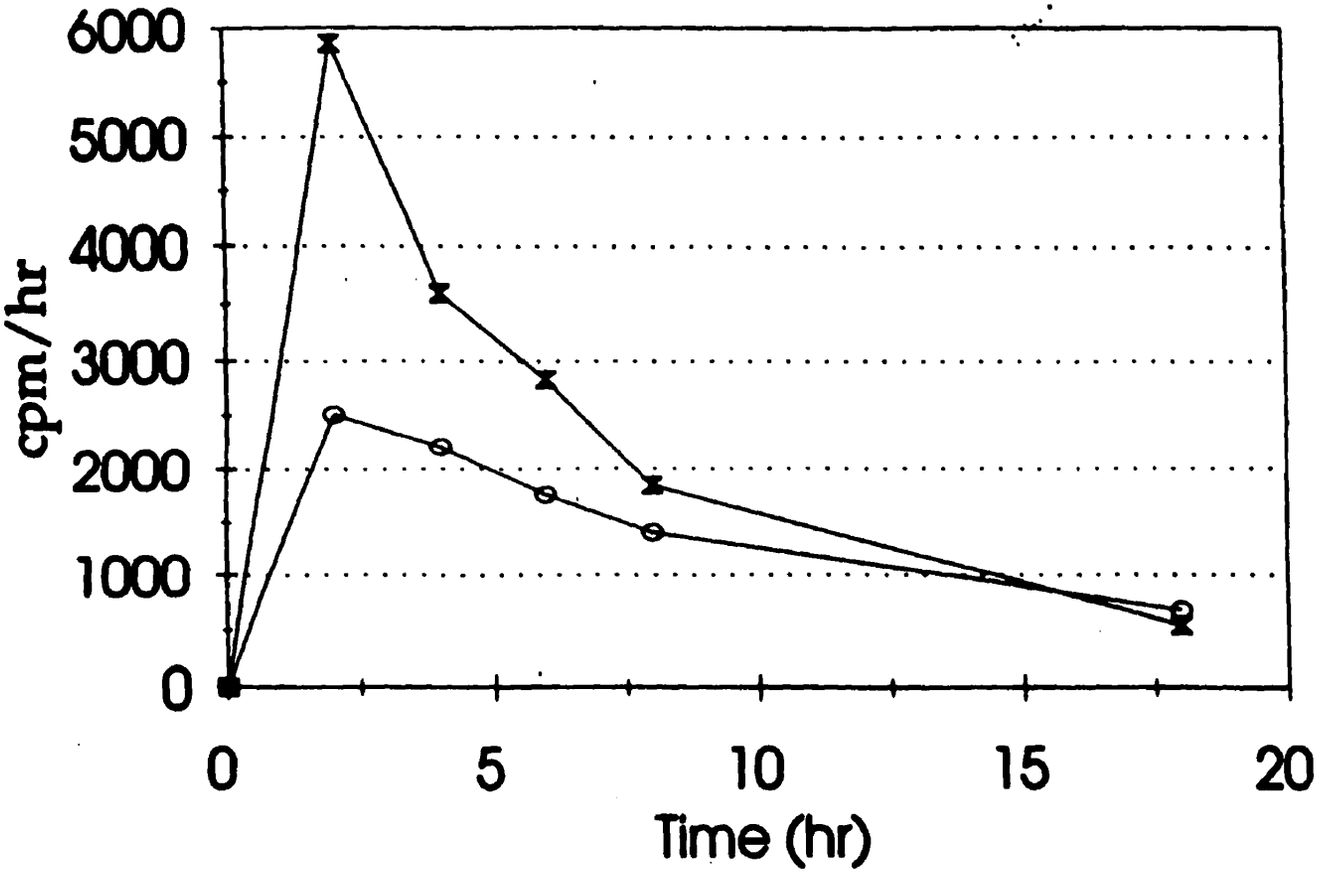
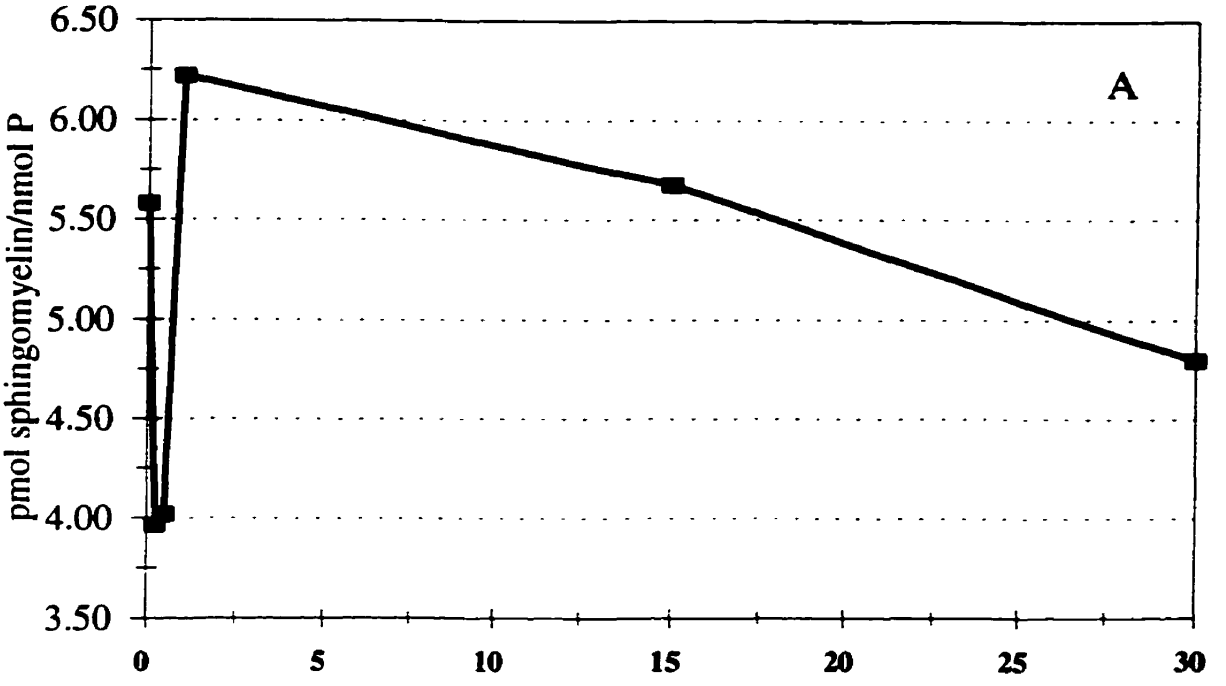

6000
5000
4000
3000
2000
1000
0
cpm/hr
0
5
10
15
20
Time (hr)

Figure 14: Sphingomyelin levels in plasma membranes and extracellular shed vesicles treated with 600 Joules/m^2


A
pmol sphingomyelin/nmol P
6.50
6.00
5.50
5.00
4.50
4.00
3.50
0
5
10
15
20
25
30

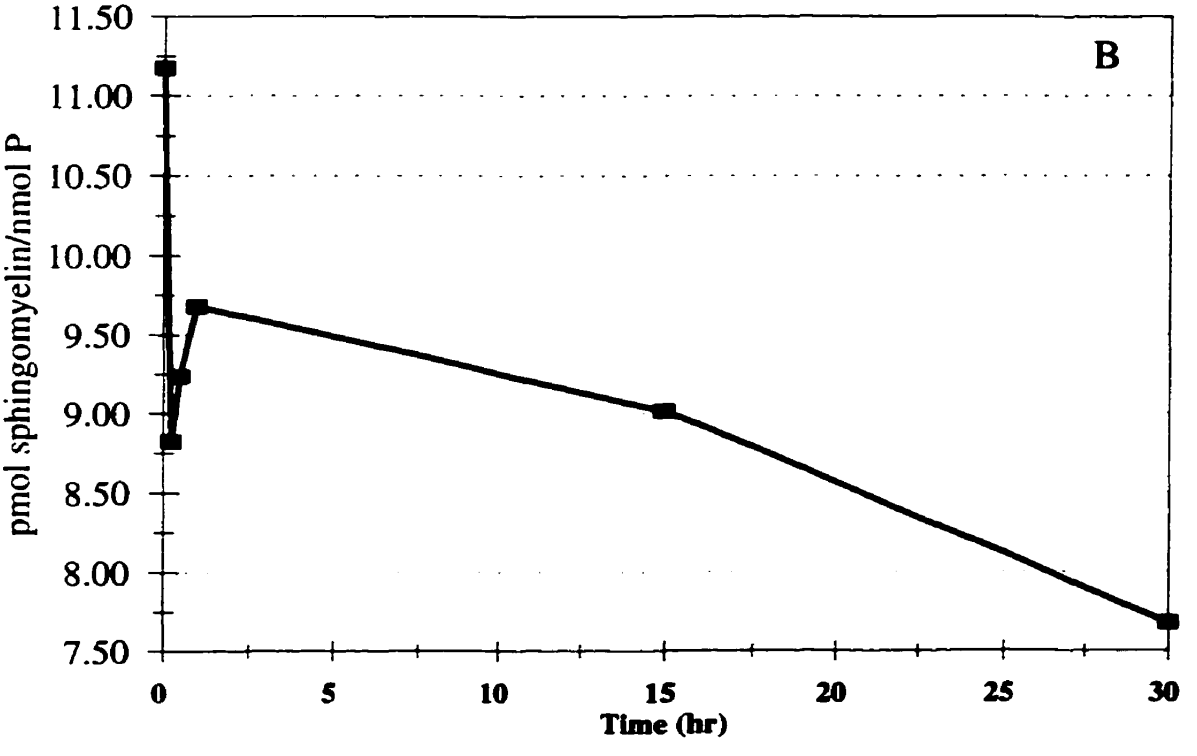
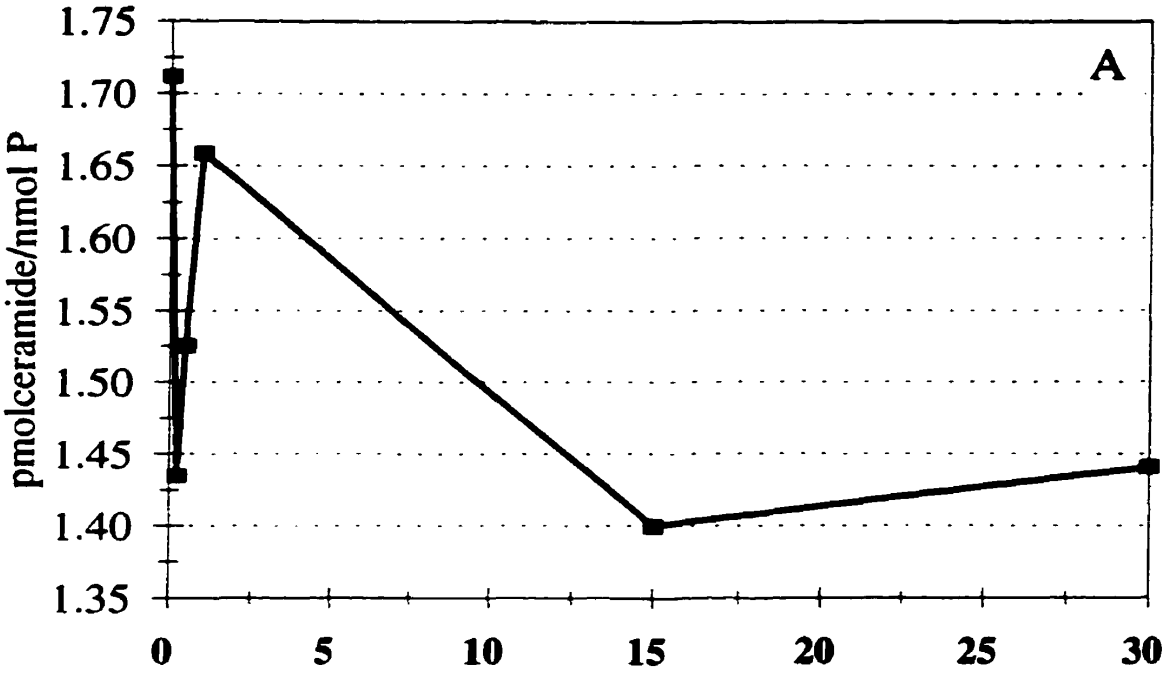
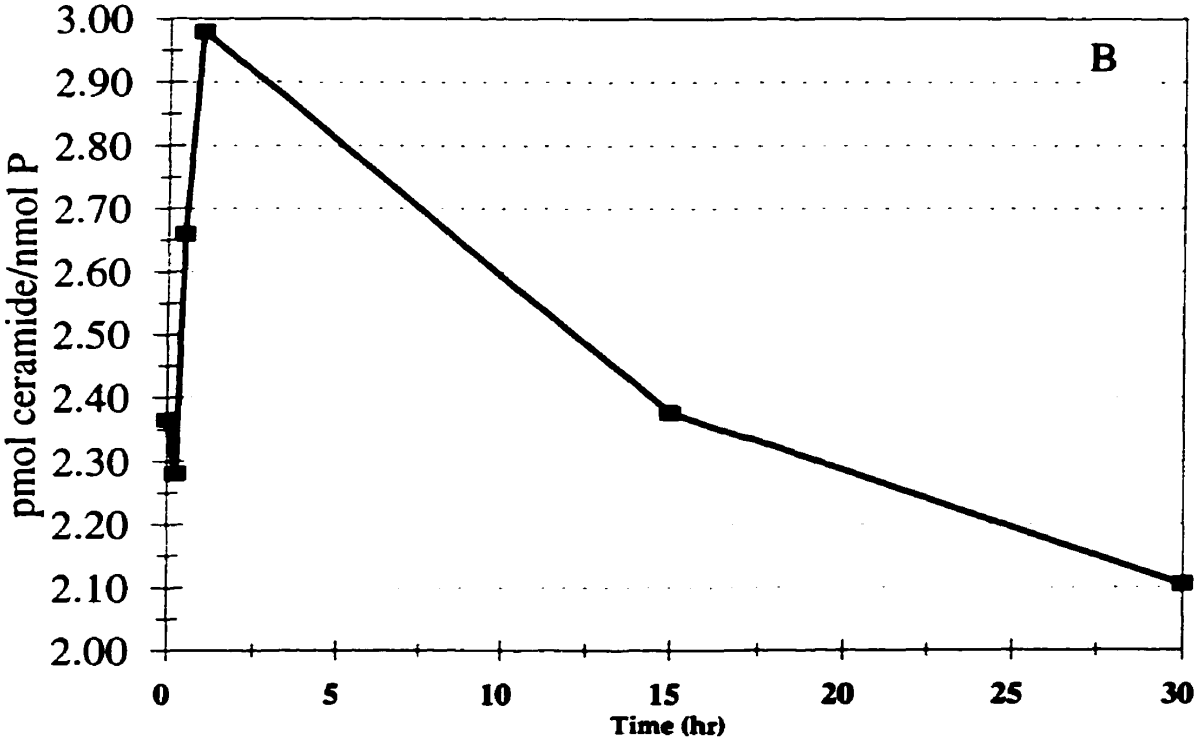

B
pmol sphingomyelin/nmol P
11.50
11.00
10.50
10.00
9.50
9.00
8.50
8.00
7.50
0
5
10
15
20
25
30
Time (hr)

Figure 15: Ceramide levels in plasma membranes and extracellular shed vesicles treated with 600 Joules/m^2


A
pmolceramide/nmol P
1.75
1.70
1.65
1.60
1.55
1.50
1.45
1.40
1.35
0
5
10
15
20
25
30


B
pmol ceramide/nmol P
3.00
2.90
2.80
2.70
2.60
2.50
2.40
2.30
2.20
2.10
2.00
0
5
10
15
20
25
30
Time (hr)

dropped from 11.4 to 8.6 pmol/nmol lipid phosphorus (figure 14A). This represents a 25% reduction of sphingomyelin relative to control samples. Over time, the level of sphingomyelin continued to decline. Provocatively, ceramide levels also decreased. As shown in figure 15A, 15 sec post-irradiation, a 15% reduction in the level of ceramide was observed in shed vesicles (1.7 to 1.45 pmol/nmol lipid phosphorous). Following this initial drop, the level of ceramide increased to 1.65 pmol/nmol lipid phosphorus in the next 45 sec and subsequently diminished over 30 min.

The effects of UV-B on the sphingomyelin and ceramide levels was also examined in plasma membranes of CHO cells following irradiation. A decrease in sphingomyelin from 5.5 to 3.9 pmol/nmol lipid phosphorus was seen 15 sec following irradiation (figure 14B). On the other hand, the level of ceramide increased 1.3 fold, from 2.3 to 2.9 pmol/nmol phosphorus at this time (figure 15B).

3.1.2 M-CSF and flt3/flk2 lignads are exfoliated on shed vesicles

The M-CSF receptor and flt3/flk2 ligand, a tyrosine kinase receptor, are expressed on hematopoietic stem cells. To investigate whether the ligands for flt3/flt2 receptors can be exfoliated on plasma membrane-derived vesicles, medium conditioned by CHO cells transfected with cDNA containing a sequence encoding for the flt3/flk2 ligand and from HS5, a human bone marrow stromal cell line, respectively, was collected and the vesicles harvested by centrifugation. The protein was detected by using polyclonal antibodies for flt3/fl2 ligand or M-CSF. Figure 16 shows that plasma membranes and shed vesicles that were collected from CHO cells and CHO conditioned medium, respectively, and solubilized, electrophoresed and reacted with anti-flt3/flk2 ligand IgG contain a protein of 32 kDal corresponding to flt3/flk2 lignad. Similarly, plasma membranes and shed vesicles

Figure 16: Flt3/flk2 ligand is present at the cell surface. Plasma membranes, and extracellular vesicles shed into serum-free medium were isolated from CHO cells transfected with cDNA encoding for flt3/flk2 lignad. Plasma membranes (PM) and shed vesicles (SV) were solubilized in detergent and subjected to western blot analysis. Note the appearance of a distinct band with molecular weight of 32 kDal in PM and SV preparations, representing the flt3/flk2 lignad.

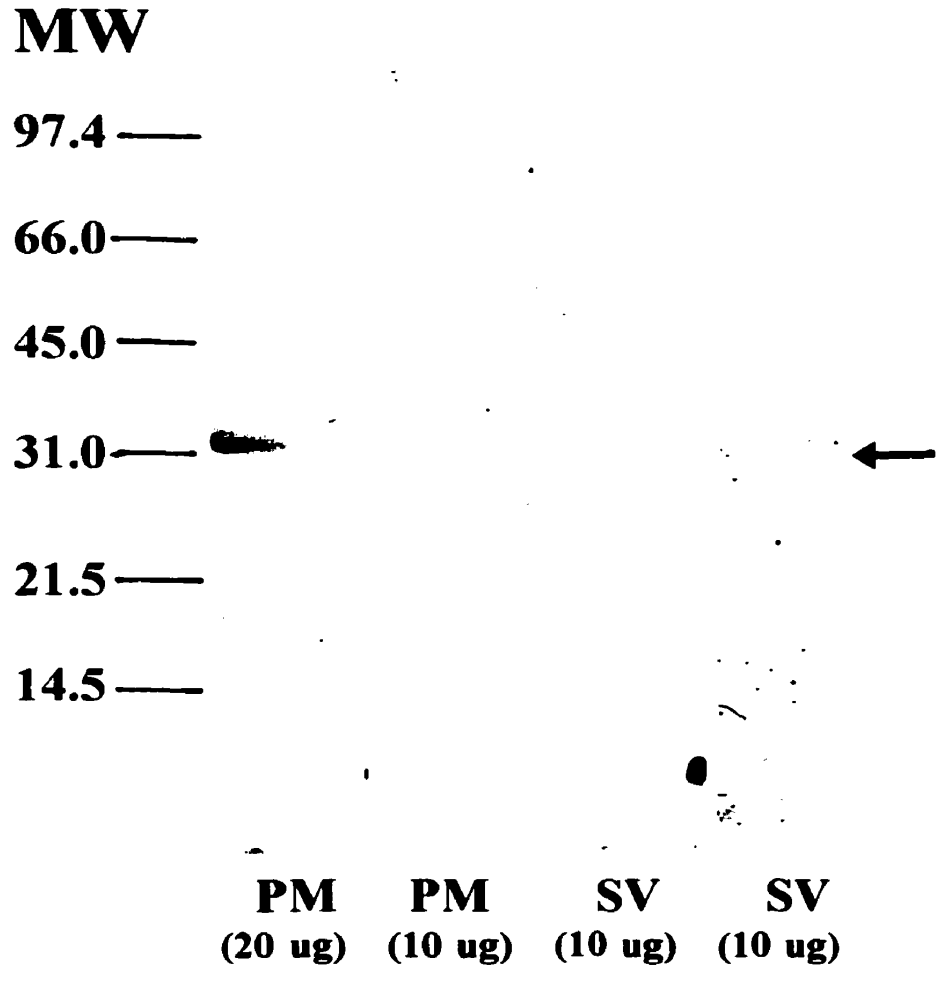

MW
97.4
66.0
45.0
31.0
21.5
14.5
PM (20 ug)
PM (10 ug)
SV (10 ug)
SV (10 ug)

Figure 17: M-CSF is present at the cell surface. Plasma membranes, isolated from HS5 and CHO (control) cells were solubilized in detergent and subjected to western blot analysis. Note the appearance of two distinct bands with molecular weights of 45 and 90 kDal for plasma membranes purified from HS5 cells (HS-5 lane) and the absence of these bands for plasma membranes purified from CHO cells (CHO lane).

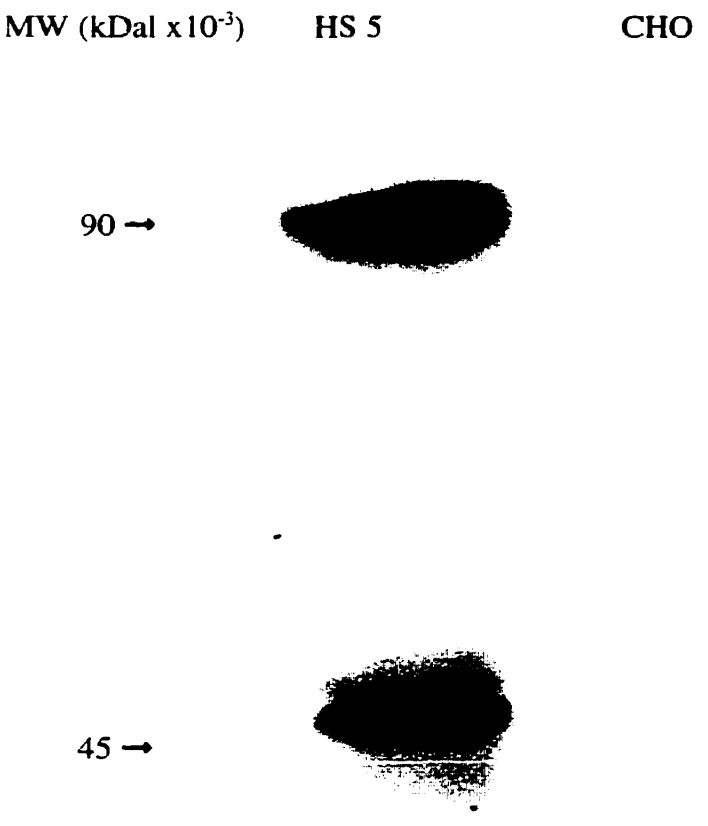
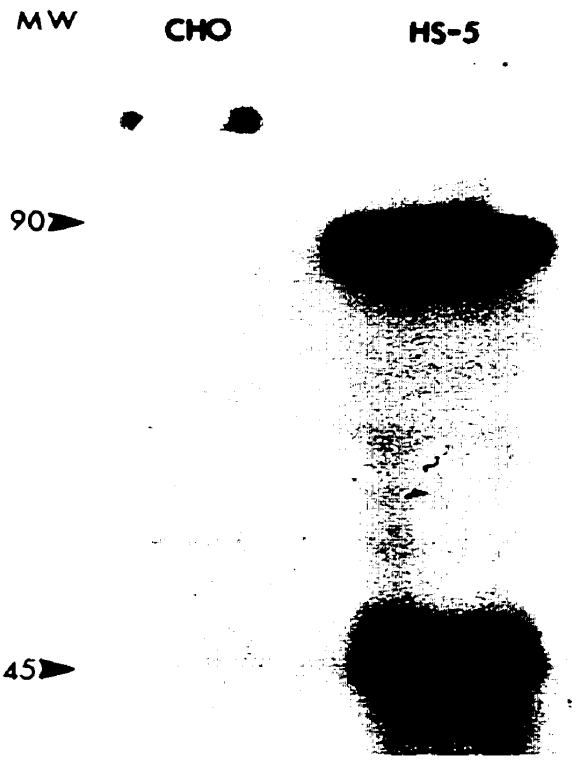

MW (kDal x10-3)
HS 5
CHO
90 →
45 →

Figure 18: M-CSF is released on extracellular vesicles. Vesicles harvested from HS5 and CHO (control) cell-conditioned media were solubilized in detergent and subjected to western blot analysis. As in figure 17, note the appearance of two distinct bands with molecular weights of 45 and 90 kDal for vesicles purified from HS5 cells (HS-5 lane) and the absence of these bands for shed vesicles collected from CHO cells (CHO lane).


MW
CHO
HS-5
90
45

and medium conditioned from HS5 cell respectively, and solubilized, electrophoresed and reacted with anti-M-CSF polyclonal IgG contained proteins of 45 and 90 kDal corresponding to the monomeric and dimeric form of M-CSF, respectively (figures 17 and 18). These results indicate that these hematopoietic ligands are released from the cells surface in association with plasma membrane derived vesicles. Implicit is the notion that vesicle-bound M-CSF and flt3/flk2 ligand can interact with target cells and induce a cellular response. This however, remains to be determined.

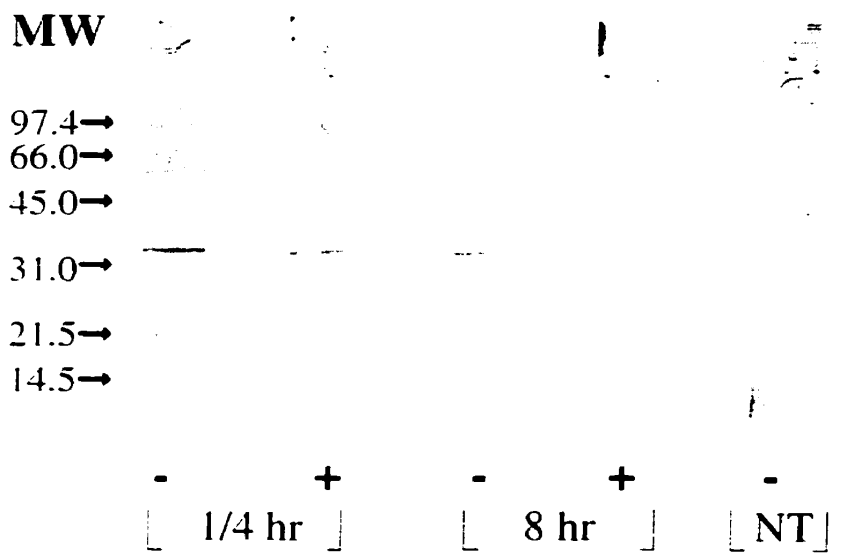
3.1.3 UV-B abrogates flt3/flk2 ligand expression at the cell surface

A variety of cytokines are either up- or down-regulated after exposure to radiation depending upon temperal sequence of UV-B treatment[283]. Distinct immune responses can be elicited *in vitro* depending upon the time of UV-B exposure. Studies indicate that expression of mBPA and M-CSF is inhibited by UV-B irradiation[159, 284]. To asses whether the expression of flt3/flk2 ligand are affected by UV-B irradiation, transfected CHO cells were treated with 600 Joules/m^2 UV-B and the plasma membranes isolated and subjected to western blot analysis.

Figure 19 shows that 15 min post-radiation treatment, there is no detectable difference in flt3/flk2 lignad (32 kDal band) expression in plasma membranes extracted with detergent. In contract, after 8 hr, no protein corresponding to the flt3/flt2 ligand is apparent on plasma membranes of CHO cells. These data suggest that transcription of the flt3/flk2 ligand gene may be altered following exposure to UV-B.

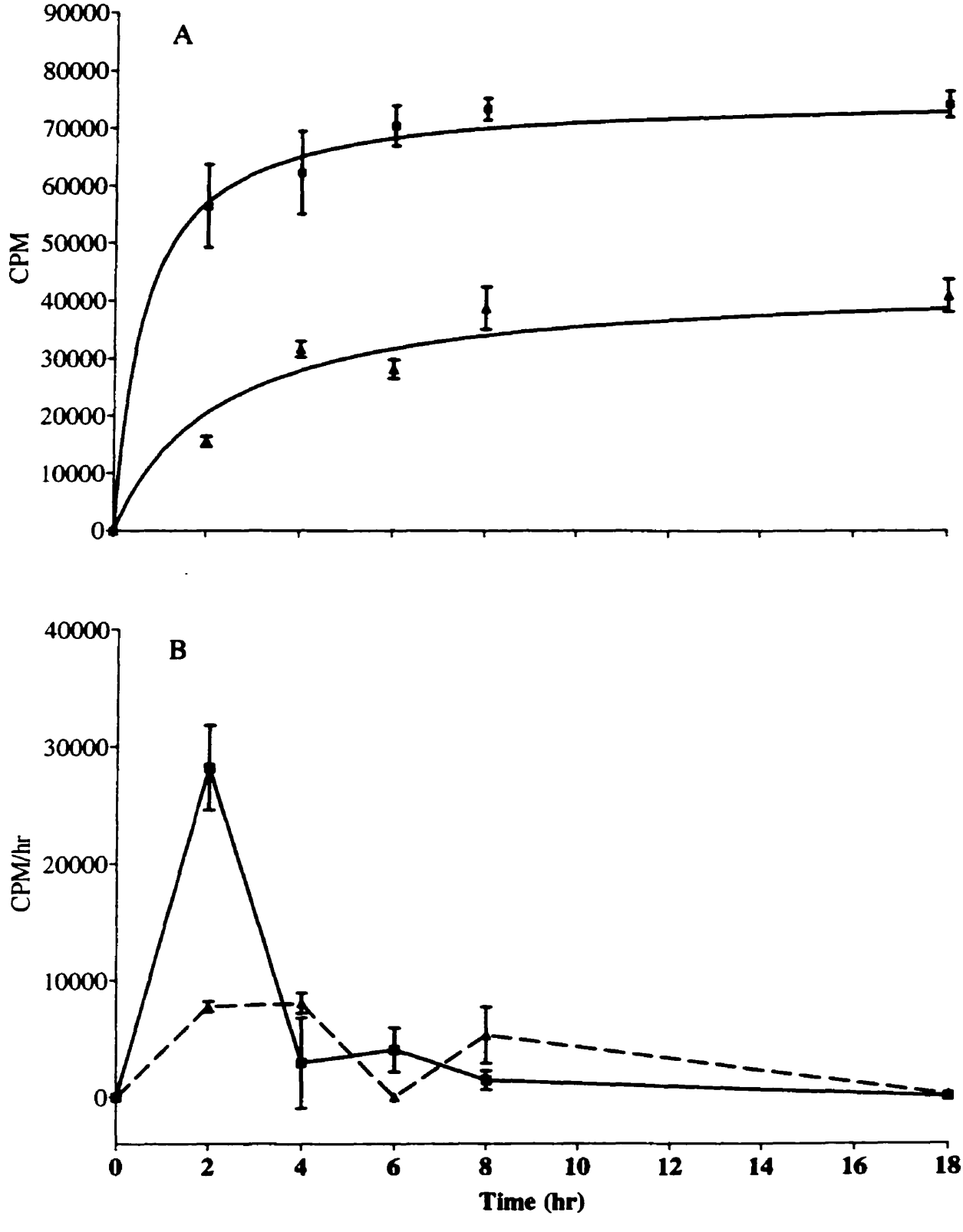
Figure 19: Inhibition of flt3/flk3 on plasma membranes by UV-B treatment. Unirradiated (-) and irradiated (+) transfected CHO cells were obtained (1/4 hr) and 8 hr following UV-B treatment (600 Joules/m^2). Flt3/flk2 ligand was detected in detergent soluble extracts of plasma membrane with polyclonal IgG, as described in *material and methods* section. Note that the flt3/flk2 lignad band (32 kDal) can still be seen on plasma membranes of CHO cells1/4 hr following irradiation, but not after 8 hr post-irradiation. Flt3/flk2 ligand is absent in nontransfected cells (NT). Lanes 1/4 hr (-, +), NT and 8 hr (-) were loaded with 10 μg protein, whereas, lane 8 hr (+) was loaded with 20 μg protein.

Effect Of UV-B On Flt3/Flt2 Protein On the PM



+ : UV-B (600 J/m^2)
- : Unirradiated CHO cells
NT: Non-transfected CHO cells

Figure 20: Kinetics of exfoliation from CX-1 and MIP-101 human colorectal adenocarcinoma cells. (A) Cumulative radioactivity (cpm) released on shed vesicles collected from 1 X 10^7surface-^{125}I-labelled CX-1 (squares) and MIP-101 (triangles) cells after indicated incubation periods was quantified by scintillation counting. (B) Rates of shedding were calculated by dividing total radioactivity (cpm) released by the incubation time. Shown are means ± SEM of 3 separate experiments.


A
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
CPM
B
40000
30000
20000
10000
0
CPM/hr
0
2
4
6
8
10
12
14
16
18
Time (hr)

Section 3.2: Bioactive Fas Receptor and Fas ligand are shed on extracellular vesicles

3.2.1 Quantitative Exfoliation from High (CX-1) and Low (MIP-101) Metastatic Potential Cell Lines:

The kinetics of exfoliation from well differentiated and high metastatic potential CX-1 cells was compared to that of poorly differentiated and low metastatic potential MIP-101 cells. As evident from figure 20A, CX-1 cells release more ^{125}I-labeled protein in association with shed vesicles over time (0 - 18 hr) than do MIP-101 cells. Cumulative radioactivity shed is 1.8 fold greater for CX-1 cells than for MIP-101 cells after 18 hr. When rates of exfoliation are compared (figure 20B), both cell lines exhibit the largest release of radioactivity (cpm) per hour after 2 hr incubation, consistent with our results of kinetic studies for shedding from normal human B cells and continuously maintained cell lines[285]. At this time, the rate of shedding from CX-1 cells was 3.6-fold greater than that of shedding MIP-101 cells.

3.2.2 Visualization of Fas on Shed Vesicles

Plasma membrane-derived vesicles pelleted from medium conditioned by CX-1 or MIP-101 cells were visualized by transmission electron microscopy, and Fas antigen was detected on their surface by immunogold particle labelling. Vesicle size and texture were heterogeneous, findings typical for shed vesicles[276]. As shown in figure 21 and figure 22, vesicles from both cell lines are heterogeneous in shape and size (ranging from 0.05 to 0.5 μm), as well as in distribution of Fas (arrowheads) on the shed vesicle surface. No difference in vesicles released from MIP-101 versus CX-1 cells was apparent by electron microscopy. However, vesicles derived from CX-1 cells (figure 22) show relatively less immunogold

particle labelling than do vesicles shed from MIP-101 cells (figure 21). The distribution of Fas on vesicles derived from plasma membranes of MIP-101 cells was typical for all fields that were examined. In contrast, vesicles shed from CX-1 cells failed to show the presence of Fas in many of the fields that were examined.

Western blot analysis of detergent solubilized MIP-101-derived shed vesicles and immunoprecipitated with anti-Fas antibody reveal two bands with apparent molecular weights of 45 and 48 kDal corresponding to differentially glycosylated Fas protein (figure 23). Treatment of the vesicle extract with N-glyconase prior to SDS-PAGE results in the disappearance of the higher molecular weight band indicating that in fact, the proteins immunoprecipitated with anti-Fas antibody are not unrelated proteins but rather are glycosylated isoforms of the same protein recognized by the antibody.

3.2.3 Quantification of Fas receptor on vesicles shed from MIP-101 cells

In order to determine the number of Fas receptor molecules released from the cell surface of MIP-101 cells on shed vesicles, the data obtained from equilibrium dialysis experiments were analyzed by Scatchard plot. Vesicles harvested from MIP-101 cells were stained with anti-Fas IgM antibody followed by treatment with FITC-conjugated anti IgM antibody and positive vesicles were sorted and collected using a flow sorter. The number of anti-Fas antibody binding sites on sorted vesicles were determined by equilibrium dialysis using ^{125}I-labeled monoclonal anti-Fas IgG antibody. As figure 24 shows, the average 3.6 Fas receptors are released on vesicles shed from the surface of MIP-101 cells.

Figure 21: Electron micrograph of immunogold-labelled Fas-bearing vesicles shed from MIP-101 cells. Fas (arrowheads) was detected with gold particle-conjugated anti-mouse anti-Fas antibody as described in *Materials and Methods*. Micrograph is at 57 000 X magnification and the inset is 68 000 X magnification; scale bar indicates 0.2 μm.

Figure 22: Electron micrographs of vesicles shed from CX-1 cells. Large arrowheads point to gold particles in association with Fas antigen on plasma membrane-derived vesicles. In (A), a few gold particles (small arrowheads) not associated with vesicles can be seen and apparently represent non-specific background. Both micrographs are at 86 000 X magnification. Scale bar indicates 0.2 μm.

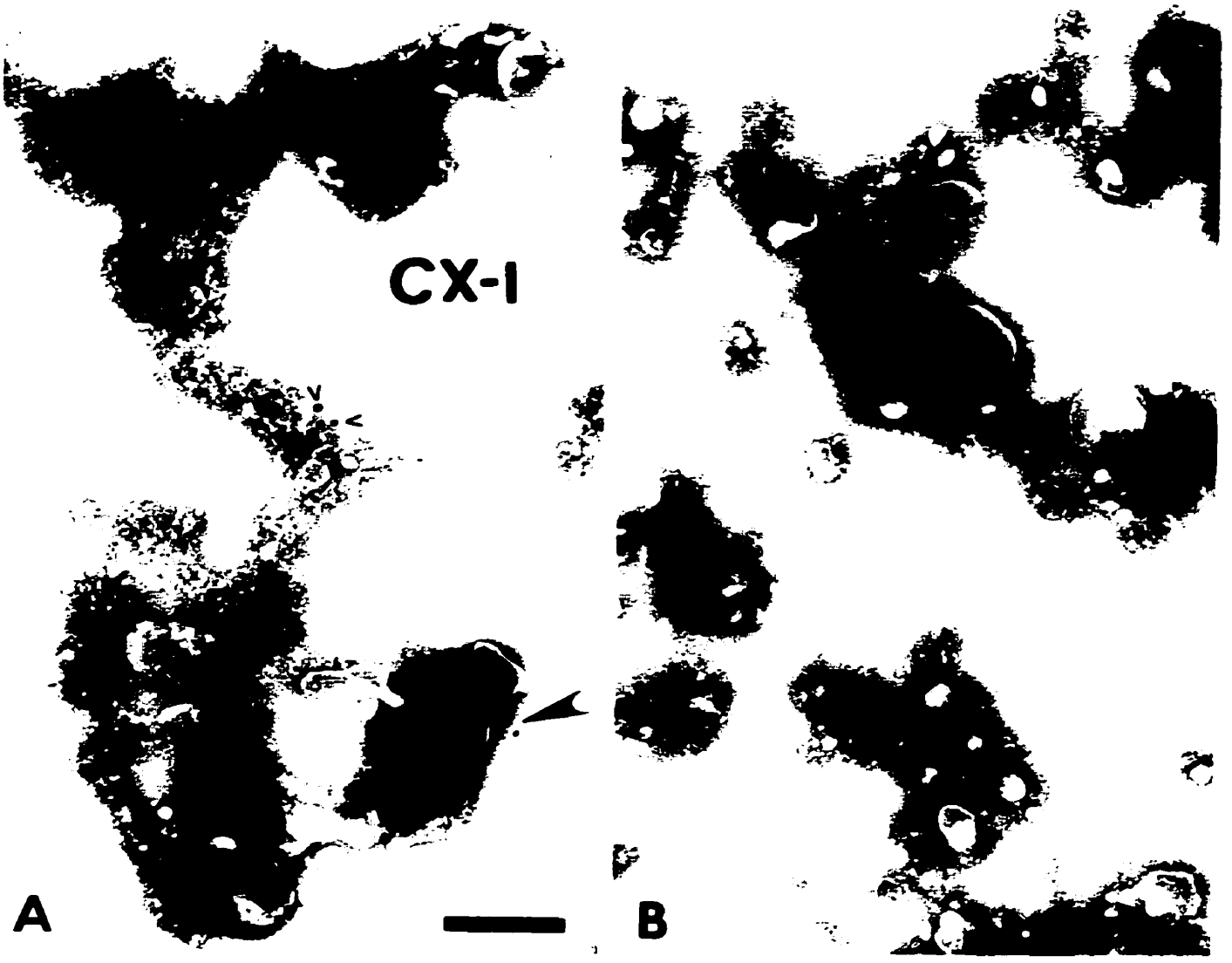
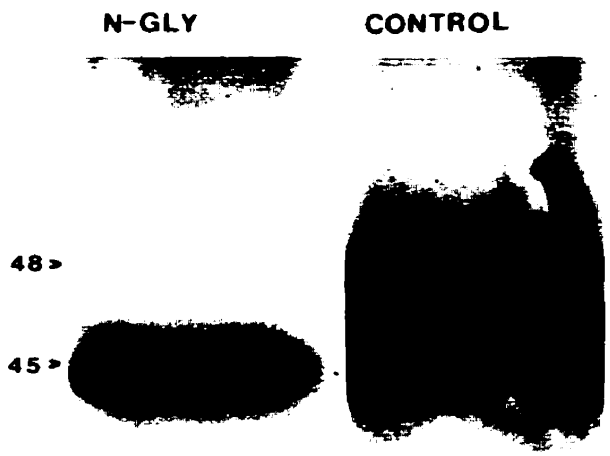

CX-1
A
B

Figure 23: Western blot analysis of solubilized extracellular vesicles harvested from MIP-101 conditioned medium. Vesicles extracted with n-octyl β-**D**-glucopyranoside and treated with N-glyconase prior to SDS-PAGE produce a single band with an apparent molecular weight of 45 kDal when probed with anti-Fas antibody (N-GLY). Vesicles extracted with detergent and subjected to SDS-PAGE without N-glyconase treatment show two bands with apparent molecular weights of 45 and 48 kDal when probed with anti-Fas antibody (Control).


N-GLY
CONTROL
48 >
45 >

3.2.4 Biosynthesis And Cell Surface Expression of Fas

CX-1 cells express less cell surface Fas than do MIP-101 cells.[286] In this study the relative distribution of Fas on the cell-surface of CX-1 and MIP-101 cells was reevaluated using flow cytometric analysis and fluorescence microscopy. Flow analysis of CX-1 and MIP-101 cells stained with IgM anti-Fas antibody and anti-IgM FITC-conjugated antibody reveals a 90-fold decrease (mean channel 5.7 for CX-1 cells verus 512.8 for MIP-101) in Fas antigen expression at the cell surface of CX-1 cells relative to MIP-101 cells (figure 25). The discontinuous ring of fluorescence on the surface of CX-1 cells (figure 26A) compared to the intense fluorescence seen on MIP-101 cells (figure 26B) confirms our initial observation that Fas appears to be downregulated in CX-1 cells relative to MIP-101 cells. Electron micrographs presented in this study revealing the presence of Fas on only very few vesicles shed from CX-1 cells suggested that exfoliation plays only a limited role in reducing the levels of cell surface Fas. This possibility prompted us to investigate rates for Fas biosynthesis in CX-1 and MIP-101 cells. Cells were labeled with ^{35}S-methionine, as described in the *Methods and Materials* section. After 6 or 16 hr incubation, the cells were lysed, and ^{35}S-labeled Fas

Figure 24: Multiple Fas receptors are released on extracellular vesicles. Fas-bearing vesicles sorted by FACS were subjected to equilibrium dialysis in which ^{125}I-labeled monoclonal anti-Fas IgG antibody was permitted to freely defuse across a dialysis membrane and bind to Fas receptors on extracellular vesicles. At equilibrium, the amount of free and vesicle-associated radioactivity was determined and the data subjected to Scatchard analysis. On average, each vesicle shed from the surface of MIP-101 cells contains 3.6 Fas receptor molecules (x-intercept, n).

n = 3.6
r/c (x 10⁵)
r
0
10
20
30
40
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

Figure 25: Flow cytometric analysis for cell surface expression Fas in CX-1 and MIP-101 cells. Cells were labeled with anti-human Fas antibody (CH.11) and then stained with FITC-conjugated anti-mouse IgG antibody as described in the *Materials and Methods* section.

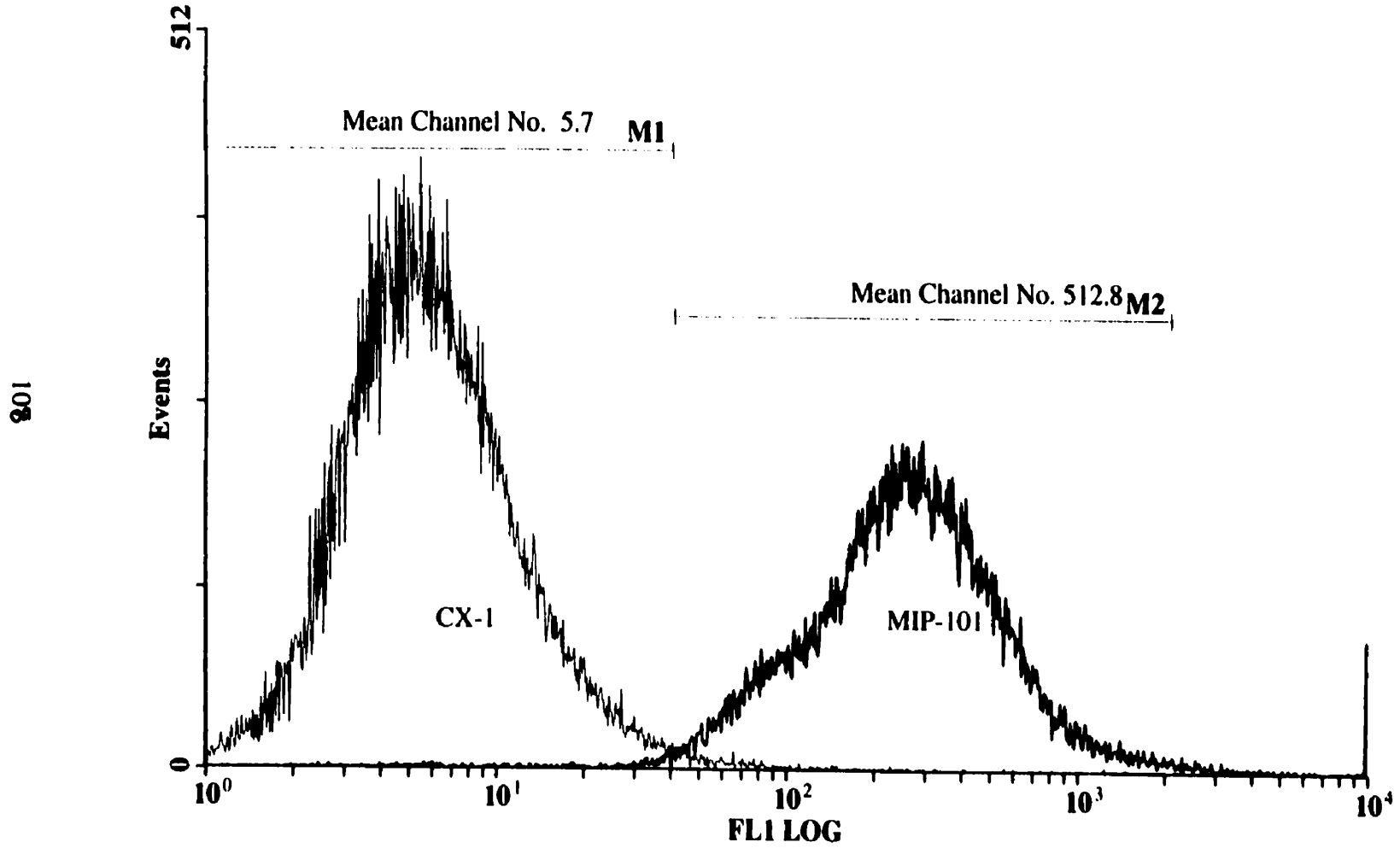
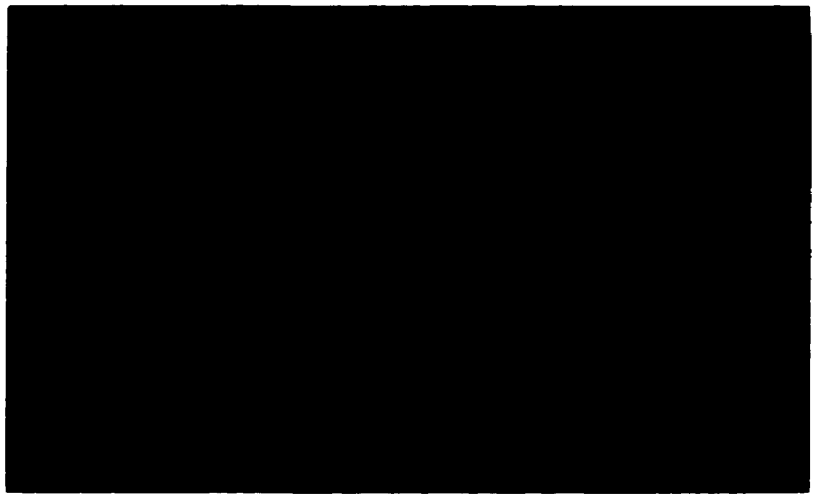

512
Mean Channel No. 5.7
M1
Mean Channel No. 512.8
M2
Events
CX-1
MIP-101
0
10^0
10^1
10^2
10^3
10^4
FL1 LOG

Figure 26: Fluorescence microscopy: cell surface expression of Fas antigen on CX-1 and MIP-101 cells Immunofluorescence microscopy indicates that CX-1 cells (A) express lower quantities of cell-surface Fas than MIP-101 cells (B). Cells were labelled with mouse anti-Fas antibody and then treated with anti-mouse FITC conjugated antibody. Note that CX-1 cells (a) show a discontinuous ring of fluorescence on their cell surface; in contrast, MIP-101 cells (b) stain very intensely with secondary antibody.



was immunoprecipitated from the lysate.

Figure 27 shows the amount of radio labeled-Fas specifically (cpm bound by anti-Fas minus cpm bound by control antibody) precipitated from lysates obtained from CX-1 or MIP-101 cells. After 6 hr, CX-1 cells synthesized 1.6-fold less Fas than did MIP-101 cells. After 16 hr incubation, CX-1 cells synthesized 3.3-fold less Fas, relative to MIP-101 cells. These results are supported by autoradiographs of ^{35}S-labeled cells showing the presence of M_r 45 000 and 48 000 proteins (corresponding to the molecular masses of differentially glycosylated Fas antigen) immunoprecipitated with anti-Fas antibody and subjected to SDS-PAGE (figure 28). Together, they suggest that reduced Fas levels on exfoliated vesicles may be related to diminished Fas synthesis and consequently, decreased Fas on plasma membranes from which shed vesicles are derived[276].

3.2.5 Biological Effects of Vesicles Shed From CX-1 and MIP-101 on Anti-Fas-Mediated Cytotoxicity

In previous experiments, we observed that CX-1 cells treated with anti-Fas antibody are more sensitive to anti-Fas mediated cell death than are MIP-101 cells, as assessed by a MTT assay (data not shown). Therefore, experiments were performed to determine whether vesicles shed from MIP-101 and CX-1 cells that were added to cells of the each type in liquid suspension altered sensitivity to anti-Fas mediated cell death. The finding of reversal of this sensitivity would suggest that Fas present on shed vesicles is biologically active, similar to bioactivity of other molecules expressed on the surface of shed vesicles.[285, 287,288]

As shown in figure 29, viability of CX-1 cells following treatment with anti-Fas alone (69.93 ± 0.72) was enhanced ($p < 0.01$) when these cells were treated with anti-Fas in

Figure 27: Synthesis of Fas by CX-1 and MIP-101 cells. CX-1 (hatched) and MIP-101 (solid) cells were cultured in methionine-free medium for 6 or 16 hr with 300 μCi ^{35}S-methionine. Cells were lysed with Triton X-100 and the lysates were immunoprecipitated sequentially with goat IgG and goat anti-Fas IgG antibody. Radioactivity in aliquots (a fifth of the total sample) of each immunoprecipitated sample was determined by scintillation. Note that Fas synthesis is greater for MIP-101 cells than for CX-1 cells ($p < 0.05$). Shown are mean values of a single experiment. Similar results were obtained in two additional studies.

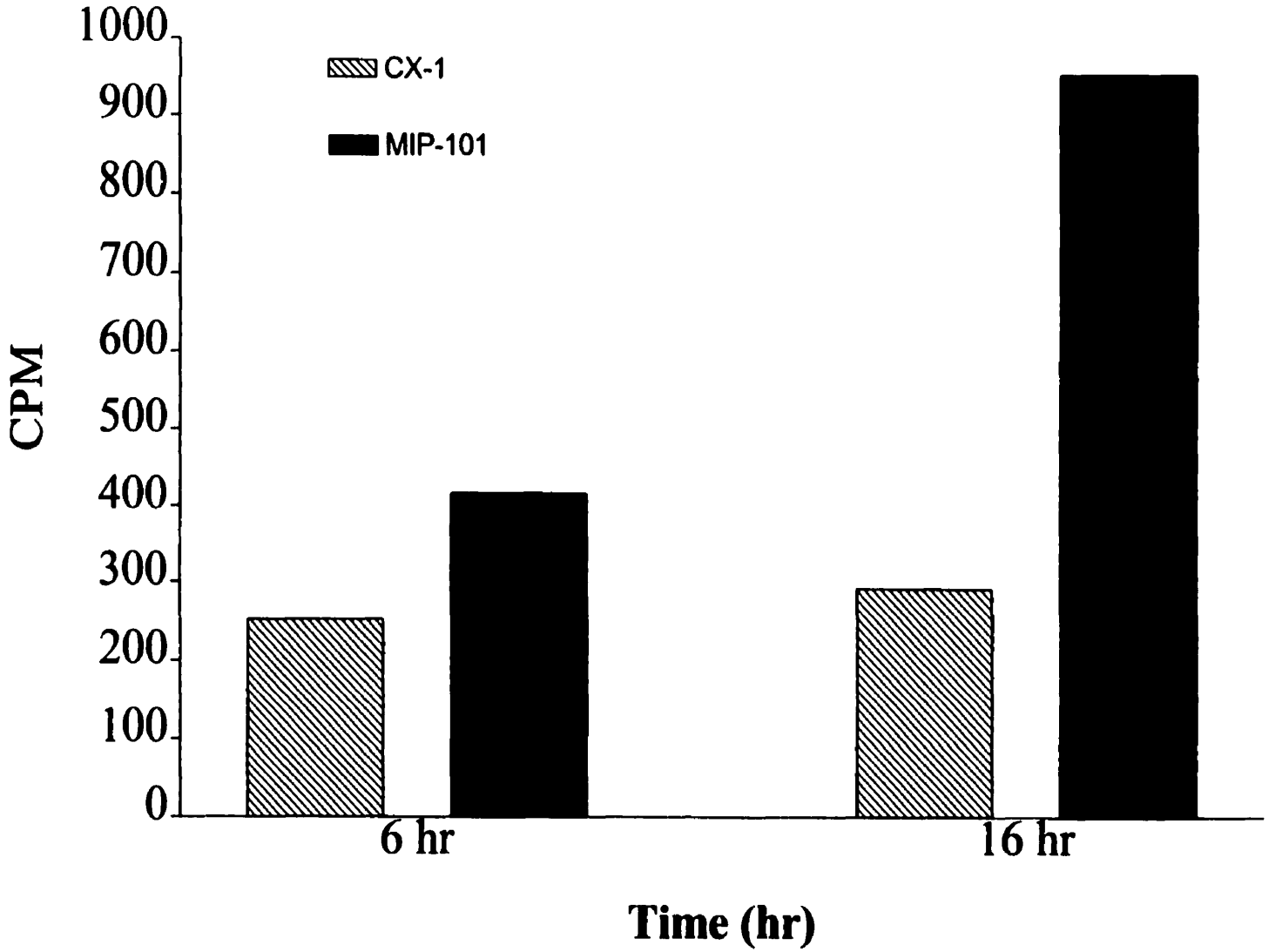

1000
900
800
700
600
500
400
300
200
100
0
CPM
CX-1
MIP-101
6 hr
16 hr
Time (hr)

Figure 28: Autoradiogram of ^{35}S-labelled, immunoprecipitated protein. Protein immunoprecipitated from lysates of MIP-101 (left) and CX-1 (right) cells incubated for 6 or 16 hr with 300 μCi ^{35}S-methionine were resolved on a 7.5 % SDS-polyacrylamide gel by electrophoresis. The autoradiogram was obtained by exposing an X-ray film to the dried gel for 5 days at -80°C.

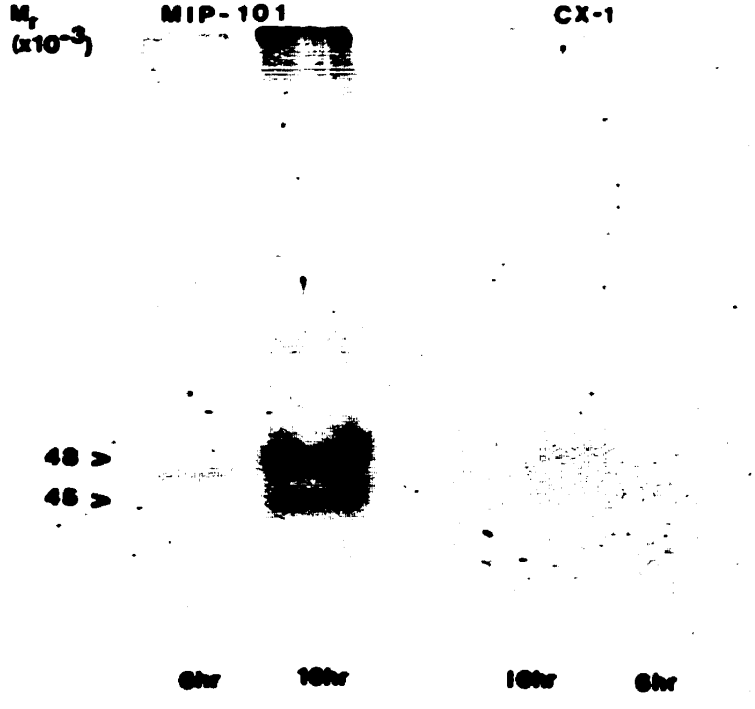
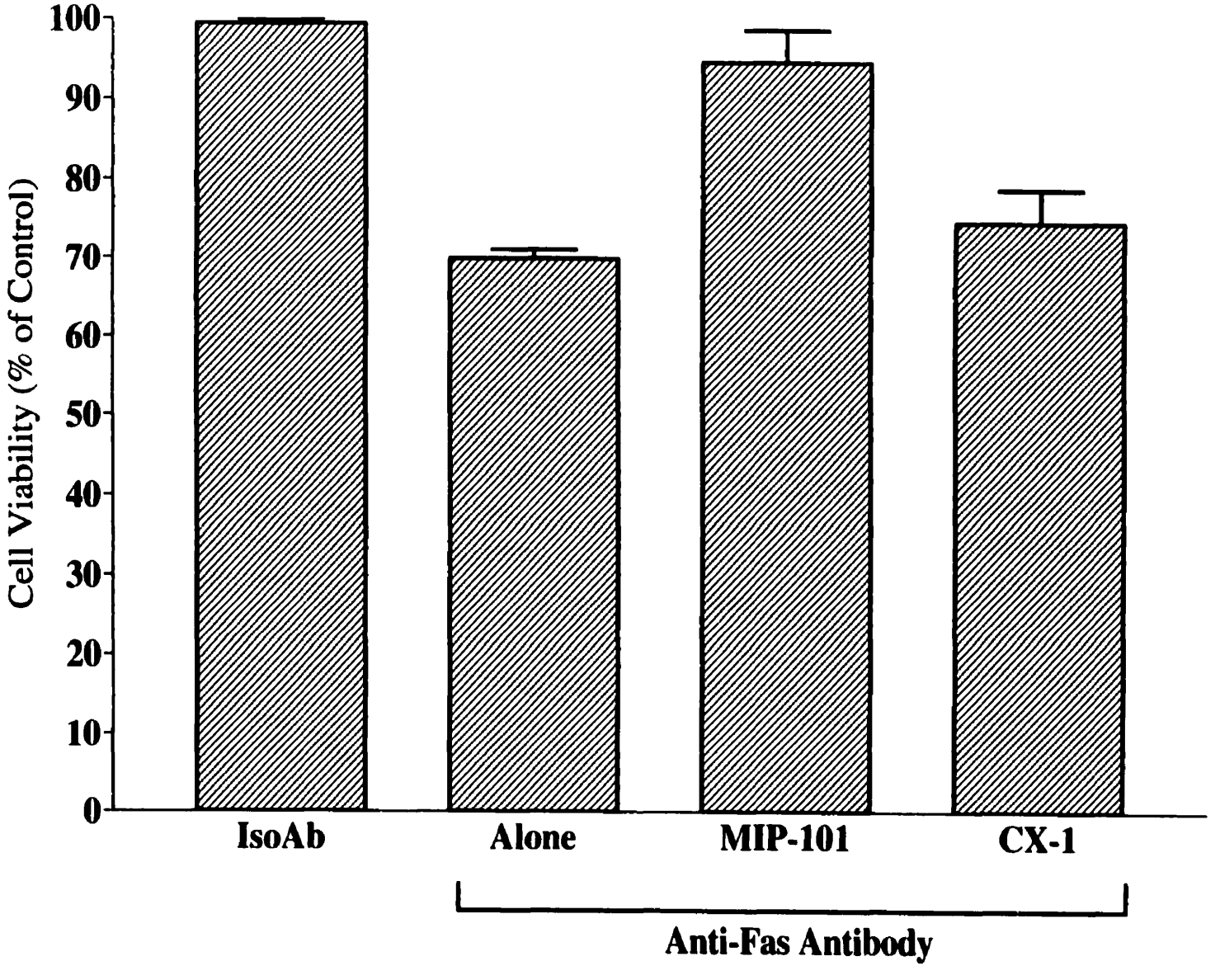

M_r (x10^{-3})
MIP-101
CX-1
46 >
46 >
6hr
10hr
10hr
6hr

Figure 29: Ability of shed vesicles to block anti-Fas-mediated CX-1 cell death. (IsoAb) Negative and (Anti-Fas alone) positive controls were established by treating 2 x 10^5 CX-1 cells with 100 ng noncytotoxic, isotype matched antibody or anti-human Fas IgM (CH.11), respectively. Bars (MIP-101) and (CX-1) represent viability of CX-1 cells treated with CH.11 in the presence of 40 μg total membrane protein prepared from vesicles shed from MIP-101 and CX-1 cells, respectively. Shown are means of 3 experiments. Note that while Fas-mediated apoptosis is abolished in the presence of vesicles shed from MIP-101 cells, apoptosis is unaffected ($p > 0.10$) by the addition of vesicles shed from CX-1 cells.


Cell Viability (% of Control)
100
90
80
70
60
50
40
30
20
10
0
IsoAb
Alone
MIP-101
CX-1
Anti-Fas Antibody

the presence of shed vesicles collected from medium conditioned by MIP-101 cells (94.87 ± 2.32). In contrast, addition of anti-Fas to CX-1 cells in the presence of vesicles collected from medium conditioned by CX-1 cells did not increase cell viability, compared to treatment of CX-1 cells with anti-Fas alone (74.23 ± 2.45 vs 69.93 ± 0.72, respectively; $p > 0.05$). These results, together with electron microscopic results revealing few Fas molecules on vesicles derived from CX-1 cells (see figure 22), indicate that Fas shed on the surface of exfoliated vesicles is bioactive, and that the amount of activity on shed vesicles correlates with the amount of antigen that can be determined by immunoelectron microscopy.

3.2.6 Visualization of FasL On shed Vesicles

To determine whether FasL is also released on shed vesicles, we examined extracellular vesicles from HuT 78 cells which are known to express high levels of FasL at their cell surface. Vesicles harvested from HuT 78 cell-conditioned medium were labeled with rabbit anti-FasL antibody and subsequently treated with anti-rabbit immunogold conjugated antibody were examined by electron microscopy. As with vesicles derived from MIP-101 and CX-1 cells, vesicles derived from HuT 78 cells are heterogenous in shape and texture. As shown in figure 30A vesicles labeled with immunogold particles (arrowheads) range in size from 0.2 to 0.4 μm. In control experiments, no labeling was observed for vesicles treated with immunogold-conjugated antibody alone (figure 30B).

Detergent solubilized plasma membranes or vesicles derived from HuT 78 cells, immunoprecipitated with anti-FasL antibody and subjected to western blot analysis reveal a 30 kDal molecular weight band (figures 31 and 32). The apparent molecular weight of this protein is similar to that of FasL,[278, 292] consistent with the notion that FasL protein on shed

Figure 30: Electron micrographs of vesicles shed from HuT 78 cells. Arrowheads point to gold particles in association with FasL on plasma membrane-derived vesicles (A). No labeling is observed if vesicles are treated with immunogold particle-conjugated antibody alone (B). Micrograph (A) is at 85 000 X magnification and micrograph (B) 47 000 X magnification .

A

B

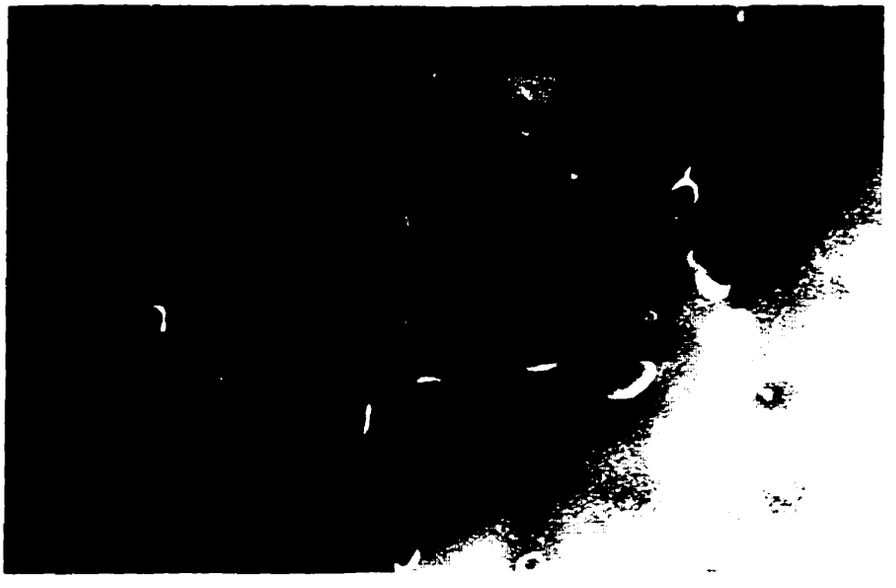


Figure 31: Fas ligand is expressed on the cell surface of HuT 78 cells. Plasma membranes isolated from HuT 78 cells, extracted with detergent and subjected to western blot analysis (as described in *Materials and Methods*) reveals a single band with an apparent molecular weight of 30 kDal (H). Similar analysis of CX-1 plasma membranes does not indicate the presence of such a band (C).

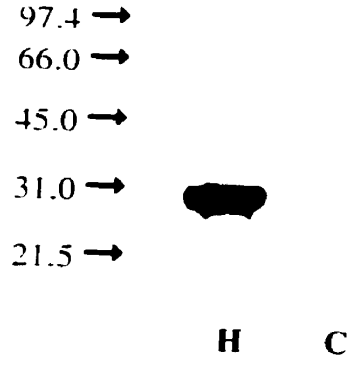
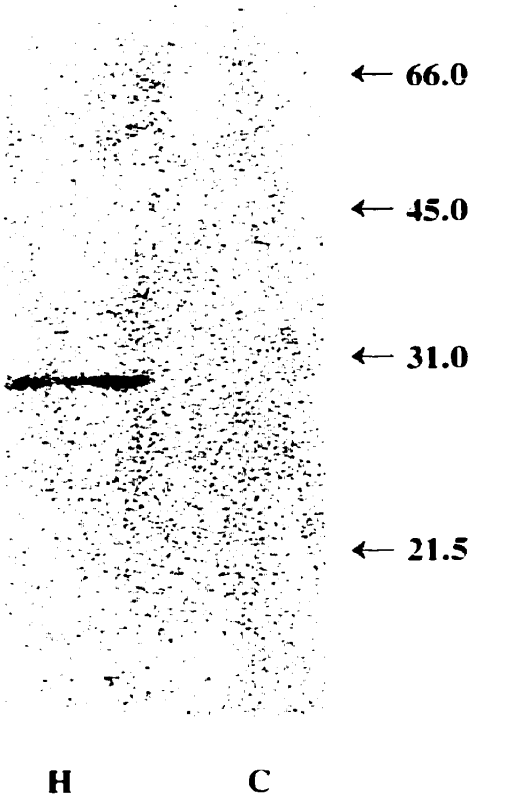

97.4
66.0
45.0
31.0
21.5
H
C

Figure 32: Fas ligand is exfoliated from the plasma membrane of HuT 78 cells on shed vesicles. Detergent extracts of HuT 78 plasma membrane-dervived extracellular vesicles, immunoprecipitated with anti-FasL antibody and analyzed by western blot produces a 30 kDal molecular weight band (H). Subjected to the same analysis, detergent extracts of vesicles collected from CX-1 conditioned medium fail to show the 30 kDal molecular weight band.

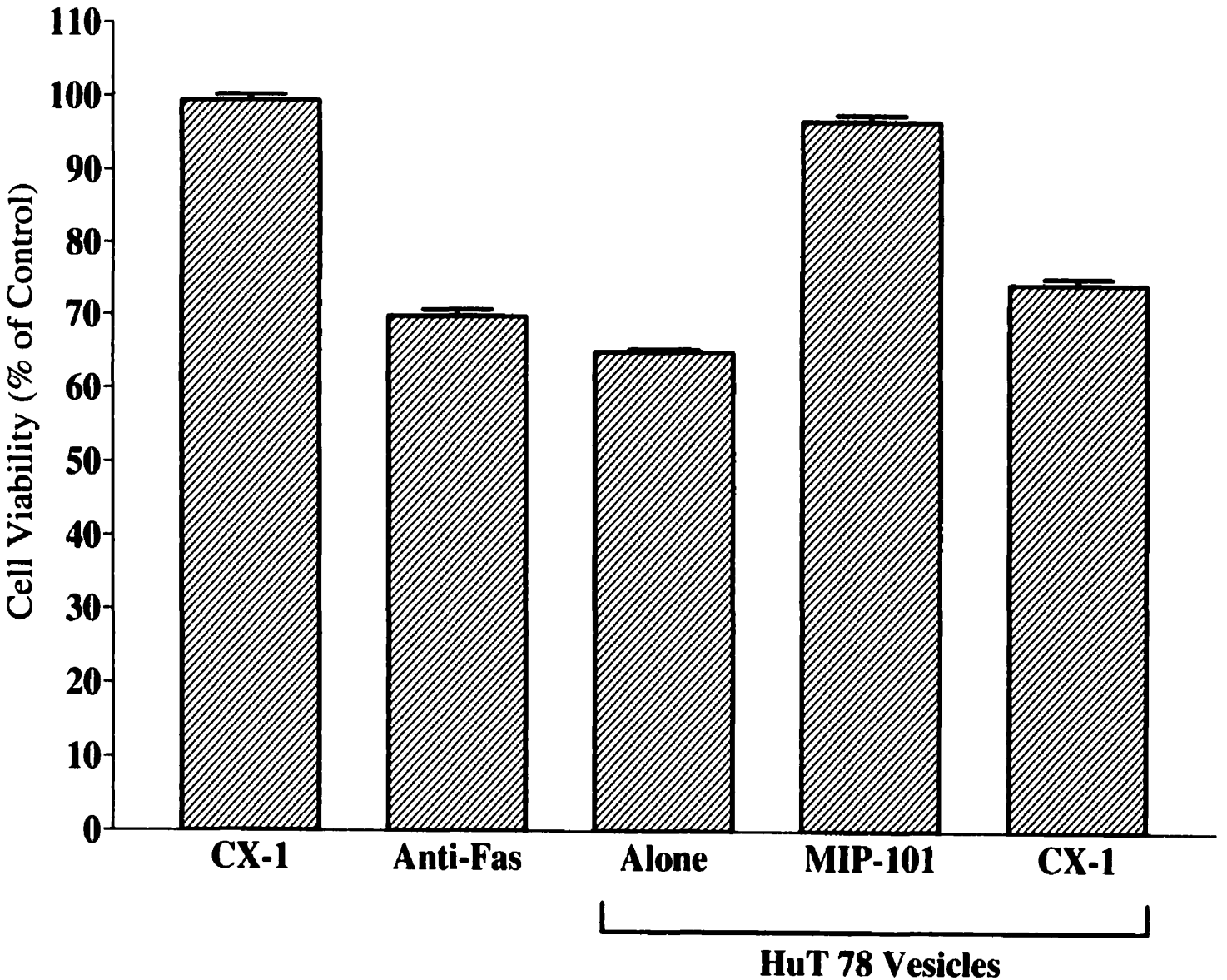

M_r (x 10^{-3})
66.0
45.0
31.0
21.5
H
C

vesicles and on the plasma membrane are identical. In contrast, FasL is not immunoprecipitated from plasma membranes or vesicles derived from the CX-1 colorectal cell line.

3.2.7 Effects Of Fas Ligand-Associated Vesicles On Cell Viability

In order to test whether, like Fas-bearing vesicles derived from MIP-101 cells, FasL-bearing vesicles derived from HuT 78 cells display biological activity, CX-1 cells were incubated with FasL-associated vesicles, and their viability determined 24 hr later using the MTT assay. As shown in figure 33, cells treated with either FasL-bearing vesicles or monoclonal anti-Fas antibody (positive control) induced comparable decreases of percent cell viability (69.40 ± 0.59 and 64.43 ± 0.63, respectively) versus 98.83 ± 0.69 percent viability ($p > 0.05$) when CX-1 cells were treated with CX-1-derived vesicles (negative control). Similar to results shown in figure 29, vesicles derived from the plasma membranes of MIP-101, but not from CX-1 cells effectively restore cell viability (97.48 ± 0.75% vs 73.93 ± 0.63%, respectively). These results provide strong evidence that FasL on shed vesicles is biologically active and that FasL interacts with its cognate receptor, Fas, when the two proteins are presented together as components of distinct populations of extracellular vesicles.

Figure 33: Ability of FasL-bearing extracellular vesicles to induce cell death. (CX-1) Negative and (Anti-Fas) positive controls were established by treating 2×10^5 CX-1 cells with 40 μg total membrane protein prepared from vesicles shed from CX-1 cells or 100 ng anti-human Fas IgM (CH.11), respectively. Bar (HuT 78 alone) represents the viability of CX-1 cells treated 80 μg total membrane protein prepared from vesicles shed from HuT 78 cells. Bars (4) and (5) represent viability of CX-1 cells treated 80 μg total membrane protein prepared from vesicles shed from HuT 78 cells in the presence of 40 μg total membrane protein prepared from MIP-101 cells and CX-1 cells, respectively. Shown are means of 3 experiments. Note that FasL-bearing vesicles and anti-Fas antibody induce similar levels of cell death ($p > 0.05$) when added to CX-1 cells. The negative effect on cell viability indued by vesicles derived from HuT 78 is reversed in the presence of MIP-101 vesicles, but remains unaffected in the presence of CX-1 vesicles.


Cell Viability (% of Control)
110
100
90
80
70
60
50
40
30
20
10
0
CX-1
Anti-Fas
Alone
MIP-101
CX-1
HuT 78 Vesicles

Chapter 4 Discussion

Section 4.1: Early events of UV-B-induced apoptosis are initiated at the plasma membrane

It is well known that nuclear DNA is a principle target in radiation-induced cytotoxicity. Radiation has been shown to act directly on genomic DNA resulting in both, single-strand and double-strand DNA breaks[289]. Particularly noteworthy, are the observations reported by Warters et al., 1977. These authors demonstrated that 60 decays/cell of ^{125}I (iododeoxyuridine) in the nucleus of CHO cells is sufficient to kill 50% of radiolabeled cells. In comparison, membrane-associated ^{125}I is surprisingly non-toxic, requiring 19 600 disintegrations/cell of the radioisotope to induce the same level of cell death[290]. Notwithstanding the role of direct interaction of radiation with nuclear DNA in cell death, recent studies indicate that at sublethal doses of ionizing radiation, apoptosis is initiated at the plasma membrane level. Treatment with lidocaine protects salivary gland cells from radiation-induced apoptosis which occurs at interphase. This finding coupled with that reported by Haimovitz-Friedman et al., 1994, showing that ionizing radiation acts directly on the plasma membrane to induce the formation of ceramide via hydrolysis of sphingomyelin strongly implicates the plasma membrane as a primary target in radiation-induced apoptosis[131].

Like ionizing radiation, UV-B has been shown to initiate apoptosis in mammalian cells as a result of DNA lesions[291, 292]. In addition, evidence suggests that like X-rays, low-doses of UV-B radiation induce plasma membrane changes. Dainiak et al, 1993, reported that lymphocytes exposed to 1200 Joules/m^2 UV-B radiation, exhibit plasma membrane

alterations including loss of villi, blunting of villi and abnormal vesiculation patterns[278, 292]. In the current study, we examined the possibility that the plasma membrane is a primary target in UV-B radiation-induced apoptosis, focusing on early events following UV-B irradiation.

The initial radiation effects at the cellular level were investigated by electron microscopy. Our results indicate that the earliest ultrastructural changes induced by UV-B radiation occurs in the plasma membrane (figure 11). These changes include the appearance of membrane vesicles in clusters that form protrusions at the cell surface and the appearance of fenestartions immediately thereafter. Since these vesicles may result from accelerated formation of vesicles or from a retention of plasma membrane-derived vesicles at the cell surface, kinetic studies on patterns of exfoliation were performed. Figures 12 and 13 indicate that cumulative shedding and rates of shedding are significantly reduced in UV-B treated CHO cells. This reduction occurs at the same time as the appearance of the plasma membrane extensions at the cell surface. Furthermore, these early events occur prior to any other morphological changes (i.e., cytoplasmic organelles) typically associated with apoptosis. Together, these results strongly suggest that exfoliation is directly inhibited by UV-B. Retained vesicles are similar to those described by Chandra and Stephani, 1981, in human lymphocytes following exposure to X-rays[86]. In addition, similar changes have been noted in studies examining the effects of ionizing radiation on rat thymocytes[293].Accordingly, an early ultrastructural change following irradiation appears to be retention of plasma membrane-derived extracellular vesicles at the cell surface.

The fate of retained vesicles is unknown. It is possible that such vesicles are

incorporated into the plasma membrane through a process of fusion[158]. Alternatively, vesicles retained at the cell surface may undergo endocytosis. Finally, it is possible that protrusions containing multiple vesicles are ultimately released from the surface. Regardles of their fate, vesicles retention potentially generate intracellular signals that lead to alteration in expression of genes that encode membrane-bound cytokines and/or induce apoptosis.

Exposure to ultraviolet light has been shown to alter the expression of a variety of plasma membrane components including autoantigens[294], cytokines[278, 295] and adhesion molecules[296, 297]. In addition, since extracellular vesicles carry biological signals that are involved in a variety of cell-cell communication pathways and processes, we investigated whether expression of flt3/flk2 lignad expressed at the cell surface of CHO cells transfected with cDNA encoding for this growth factor is (i) influenced by UV-B irradiation and (ii) whether flt3/flk2 lignad is shed on plasma membrane-derived vesicles. This cytokine, in synergy with other growth factors such as KIT-ligand IL-3 and IL-6, promotes the survival and proliferation of early hematopoietic progenitor cells[298]. Our results (figure 19) clearly indicate that 8hr following UV-B treatment, the protein is no longer present at the cell surface. We conclude that expression of flt3/flk2 lignad is decreased by ultraviolet B irradiation. Whether reduced flt3/flk2 lignad levels is the result of transcriptional, translational or posttranslational regulation is unknown. Additional studies are required to determine the mechanism by which UV-B radiation alters cell surface expression of flt3/flk2 ligand. Consistent with other studies suggesting that cell surface constituents are exfoliated from the plasma membrane on shed vesicles, figures 16 and 18 indicate that flt3/flk2 ligand as well as, M-CSF are released from the surface of CHO cells as components of extracellular

vesicles. Whether UV-B irradiation decreases the expression of these growth factors shed on vesicles from irradiated cells remains to be elucidated. However, precedence for this possibility was provided by Dainiak and Sorba, 1990. In their study, these authors reported that mBPA-bearing shed vesicles from lymphocytes irradiated with 7200-3600 Joules/m^2 express dose-dependent reduced activity, relative to activity on vesicles shed from unirradiated cells.

Data presented in this document suggest that UV-B radiation has profound effects on the plasma membrane. In addition to reducing the level of vesicles released from the cell surface of cells, UV-B abrogates expression of some cytokines that are normally found on shed vesicles and plasma membrane. It is entirely possible that vesicles retained at the cell surface generate or concentrate a second messenger molecule (e. g., ceramide via radiation-induced hydrolysis of sphingomyelin) that, ultimately, is responsible for initiating apoptosis.

Interestingly, mounting evidence suggests that epithelial cells as well as, endothelial cells undergo apoptosis (anoikis) when they lose contact with extracellular matrix[299, 300]. Ruoslahti and Reed, 1994, have hypothesized that this phenomenon is a protective mechanism against neoplasia, preventing epithelial cells from colonizing elsewhere[301]. Since cell attachment to the extracellular matrix occurs via cell surface adhesion molecules[302] (i. e., β1 integrins), down regulation of these anchor molecules resulting from UV-B irradiation may provide an alternative mechanism for radiation-induced apoptosis as a consequence of weak cell-matrix interactions. This possibility is particularly attractive in view of recent results reported by Gniadecki et al., 1997. These authors provide evidence that, in vitro, UV-B-induced apoptosis is blocked in keratinocytes adhering via β1 intergins[303]. Hence,

decreased expression of adhesion molecules at the cell surface may greatly influence cell survival following exposure to ultraviolet radiation.

Cytokines play a critical role in regulating the function of the skin immune responses. For example, a recent study suggests that M-CSF, and GM-CSF, increase the levels of class II MHC antigen mRNA in Langerhans cells[304]. The same investigators also demonstrate that c-fms, the gene encoding for M-CSF receptor, is expressed in Langerhans cells, implying a physiological importance of M-CSF in Langerhans cells, in vivo. Keratinocytes express a large number of cytokines, including the membrane-bound form of M-CSF[305] and GM-CSF[306]. Our observations that (i) flt3/flk2 ligand is down regulated at 8 hr following UV-B treatment, that (ii) both flt3/flk2 ligand and M-CSF are exfoliated on shed vesicles from the cell surface of CHO cells and that (iii) vesiculation is impaired by UV-B irradiation have potential implications regarding the exposure of individuals to ultraviolet radiation. It is conceivable that UV-B radiation curtails the availability of M-CSF to Langerhans cells either by, preventing its expression at the cell surface in keratinocytes, inhibiting the release on plasma membrane-derived extracellular vesicles bearing M-CSF, or both. Hence, UV-B-induced downregulation of some keratinocyte-derived cytokine availability may contribute to immunosuppression induced by sun exposure.

Section 4.2: Fas ligand and its cognate receptor are shed on extracellular vesicles:

Survival and differentiation signals are transmitted among eukaryotic cells via interactions of growth factors released by effector cells in the form of (i) soluble cytokines and/or (ii) shed vesicle-bound growth factors, with cognate receptors expressed on the target cell surface[158,307]. Alternatively, membrane-bound growth regulators are presented to cognate

receptors via direct, intercellular interactions, a process referred to as "juxtacrine" communication[308]. Comparably, cell death (i. e., apoptosis) signals are also triggered by soluble factors (e. g., tumor necrosis factor-α), perforin-containing vesicles, or by direct physical interactions of Fas ligand expressed on cytotoxic T lymphocytes and NK (natural killer) cells with Fas receptor present on target cells.[309,310,311]

In this study, our finding that bioactive Fas and FasL are shed on vesicles derived from the plasma-membrane of MIP-101 cells and HuT 78 cells, respectively is in complete accord with findings reported previously illustrating that growth factors, mBPA, M-CSF and flt3/flk2 ligand are released on vesicles where they show biological activity. The release of cell-surface molecules by effector cells on shed vesicles provides another mechanism for intracellular communication that is essentially a compromise between the release of soluble but diffusible factors able to trigger their receptors on target cells which are not necessarily adjacent to the effector cell, and juxtacrine activity where long lasting stimulation can be achieved only through direct and physical contact between effector and target cell. Ligand-bearing vesicles permit long range interaction while still maintaining effector molecules concentrated on a membrane surface thereby restraining their dilution in the pericellular environment.

There is evidence to suggest that the mechanism by which proteins are released on extracellular shed vesicles (exfoliation) from the cell surface is dependent on intracellular processes, including mRNA synthesis and translation as well as post-translational modification[161]. Since the release of proteins on vesicles shed from the cell surface requires energy and active cell metabolism, and occurs from distinct regions of the plasma membrane,

exfoliation is a directed (rather than random) process[312,313].

Exfoliation serves not only to limit the surface area-to-volume ratio of cells but also to maintain tissue homeostasis. For example, membrane bound burst-promoting activity, an erythroid-directed growth factor, is shed on vesicles derived from plasma membranes of both lymphocytes and monocytes, and stimulates the proliferation of human marrow progenitor cells[161, 162]. Recent evidence suggests that other cytokines, including macrophage colony-stimulating factor are exfoliated and interact with target cells as extracellular vesicle-bound growth regulators[285,314]. Moreover, transferrin receptor[315], C3 component of the complement system, fragment crystallisable (Fc) domain of antibodies[316] as well as class I and class II major histocompatibility (MHC) antigens[166, 317] are also shed on plasma membrane-derived vesicles where they are biologically active. It has been suggested that the survival and metastatic propensity of many tumor cell types may be enhanced by the down regulation of cell-surface tumor antigens thereby allowing these cells to avoid immune recognition and destruction[318,319]. Studies showing that, in some cases, high metastatic potential cell lines shed higher levels of plasma membrane-derived vesicles relative to their low metastatic potential counterparts have strengthened this concept[320,321]. The shedding patterns we observed in this study suggesting that high (CX-1) metastatic potential cells release more plasma membrane-derived vesicles than the low (MIP-101) metastatic potential cell line (figure 20) collaborate these latter findings. Whether the increased level of shedding shown by CX-1 cells protects against imunnorecognition and facilitates their metastasis remains to be investigated, however, the possibility is an intriguing one.

Speculation in the literature about the physiological significance of soluble Fas in the

serum of patients with malignant tumors has included the hypothesis that its presence potentially hinders the apoptotic cell death of cancer cells initiated by T and NK cells. This model was proposed by Hughes and Crispe who demonstrated that a soluble isoform of murine Fas that is generated by alternative splicing of Fas mRNA physiologically limits apoptosis that is induced by Fas-Fas ligand association[322]. Since then, several reports have identified the presence of soluble Fas in the plasma of patients with hematologic and nonhematologic malignancies which have served to fuel this hypothesis.

Despite accumulating evidence for circulating soluble Fas in patients with malignant disorders, the source of soluble Fas is not always clear. In a recent publication, Knipping et al., reported that soluble Fas harvested from condition medium of B-lymphoblastoid cells migrates to the same position as the membrane-bound form of Fas when analyzed by SDS-PAGE[323]. These authors concluded that soluble Fas was not generated by simple proteolytic cleavage from the cell surface, but rather that soluble Fas was directly secreted into the medium, possibly because it lacks a transmembrane domain[274, 323]. Based on precedence for shedding in vivo[324] combined with the immunoEM data (figure 21) revealing the presence of Fas on vesicles shed from the MIP-101 cells we propose the possibility that soluble Fas detected in patients with B-lymphocytic leukemia, as described by Knipping et al., may be vesicle-bound Fas shed from leukemic cells. Indeed, the method employed by Knipping et al., to isolate soluble Fas parallels our method (i. e., ultracentrifugation) used to harvest shed vesicles from conditioned medium in this study, as well as that used to collect vesicles from lymphocyte conditioned medium in previous studies.[312,276] Reinforcing this hypothesis is the fact that plasma membranes (figure 28) or vesicles (figure 23) derived from MIP-101 cells,

extracted with detergent and immunoprecipitated with anti-Fas antibody reveal bands of similar molecular weights (45 and 48 kDal) when subjected to western blot analysis.

Flow cytometric analysis suggests that CX-1 cells express lower quantities of cell-surface Fas when compared to MIP-101 cells[325]. This observation is consistent with immunofluorescence data (figure 26) presented in the current study. Here, we further show that CX-1 cells synthesize Fas at a lower rate than do MIP-101 cells (figure 27). Provocatively, low metastatic potential MIP-101 cells are more resistant to anti-Fas antibody-mediated apoptosis than are CX-1 cells under culture conditions[325]. This apparent paradox may be explained by our finding that MIP-101 cells release plasma membrane-derived vesicles containing a greater level of Fas than that present on vesicles shed from the surface of CX-1 cells (figures 21 and 22), even though shedding rates are lower for MIP-101 cells (figure 20). Thus, MIP-101 cells may release vesicles bearing high levels of Fas that effectively compete with cell surface Fas for binding of anti-Fas antibody, thereby blocking Fas-mediated cell death. In such a scenario, anti-Fas antibody added to a culture of MIP-101 cells is engaged by vesicle-associated Fas and despite elevated expression of cell surface Fas (relative to CX-1 cells), MIP-101 cells escape anti-Fas antibody-mediated apoptosis. This model predicts that extracellular vesicles released from CX-1 cells having low levels of Fas should not effectively "protect" against anti-Fas antibody-induced apoptosis, a result that was observed in our studies (figure 29). Moreover, vesicles derived from MIP-101 cells, but not from CX-1 cells inhibited CX-1 cell death induced by vesicles derived from HuT 78 cells (figure 33), an activated T cell line which expresses FasL (figure 31). These results suggest that, like Fas expressed on MIP-101 cells, FasL expressed on HuT 78 cells is released on

plasma membrane-derived shed vesicles and is present in a bioactive conformation capable of interacting with vesicles-bound Fas or cell-surface Fas to induce cell death. The presence of FasL on vesicles shed from HuT 78 cells was confirmed by western blot analysis (figure 32).

This study provides evidence for an alternative mechanism for the release of Fas and its natural ligand from the surface of cells derived from human cancers. Our results show that a colorectal carcinoma cell line (MIP-101), in vitro, sheds Fas on plasma membrane-derived vesicles which are capable of neutralizing anti-Fas antibody- as well as FasL-mediated cell death. These findings add to the growing body of evidence suggesting that Fas released from the cell-surface of tumor cells can potentially act to suppress NK and T cell induced cytotoxicity.

Recently, Sato et al. have shown that lymphoma cells express and release soluble FasL into the blood of patents that is directly responsible for liver damage and pancytopenia[326]. This report is consistent with an earlier publication by Tanaka et al, demonstrating that individuals with large granular lymphocytic leukemia or NK cell lymphoma are susceptible to systematic tissue damage resulting from the action of a metalloprotease which cleaves cell-surface FasL and releases a soluble variant of the ligand into the blood of these patients[327]. Moreover, cell surface expression of FasL in melanoma cells has been directly implicated with induction of apoptosis in tumor-infiltrating T lymphocytes[328]. Under these circumstances, it is unknown whether apoptosis results from direct effector-tumor cell interaction or involves triggering of Fas receptor on activated T cells via soluble FasL released by melanoma cells. Alternatively, we have shown that FasL

can also be released from the cell-surface on shed vesicles where it is biologically active. Whether this occurs with malignant cells in vivo remains to be investigated. Notwithstanding, Liepins et al., 1978, noted that lymphocyte-tumor cell interactions induce formation of shed vesicles which detach from the target cell surface and specifically adhere to the apposing lymphocyte. Electron micrographs of effector-target cell conjugates unequivocally reveal the presence of barrier composed of extracellular vesicles between lymphocytes and target cells, suggesting a potential cancer cell escape mechanism[329, 330].

In summary, our data indicate that Fas and FasL are shed from the cell surface on extracellular vesicles. Vesicle-bound Fas competes with membrane-bound Fas on the cell surface of colorectal carcinoma cells for anti-Fas antibody and FasL present on shed vesicles blocking Fas-induced cell death. FasL-bearing vesicles, derived from HuT 78 cells, an activated human T cell line, triggers cell death in Fas-expressing cells. We propose that vesicle-bound Fas released from cancer cells binds to membrane-bound FasL on NK and/or T cells and provide a *defense* mechanism against Fas/FasL-mediated apoptosis. The release of FasL on extracellular vesicles from malignant cells potentially binds to Fas receptor present on NK and/or activated T cells to induce apoptosis and thus, provide an *offense* mechanism through which tumor cells may escape immunodistruction.

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CONTRIBUTION TO ORIGINAL RESEARCH

1. Ultraviolet-B radiation suppresses the expression of flt3/flk2 ligand on the cell surface of CHO cells.
2. Ultraviolet-B radiation alters the plasma membrane ultrastructure inducing protrusions and fenestrations on the cell surface. Such alterations indicate that the plasma membrane may represent a primary target in ultraviolet-B-induced cell damage.
3. Ultraviolet-B changes the exfoliation patterns of CHO cells. Both cumulative shedding and rate of shedding are attenuated following exposure to UV-B, features which potentially represent early events in ultraviolet-B-induced apoptosis.
4. Growth regulatory molecules, flt3/flk2 ligand, M-CSF, FasL and Fas receptor are shed from the cell surface of CHO cells and tumor (MIP-101 and CX-1) cell lines on extracellular plasma membrane-derived vesicles.
5. Fas receptor and Fas ligand released on shed vesicles are present in a bioactive configuration. Fas-bearing vesicles prevent apoptosis in tumor cells (CX-1) when co-incubated with anti-human Fas antibody, while Fas ligand-bearing vesicles induce apoptosis in CX-1 tumor cells.
6. Fas and Fas ligand recognize each other when expressed on the surface of extracellular vesicles.