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CELL INJURY AND APOPTOSIS

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Abstract

Various forms of cellular injury, whether induced by immune effector cells, aberrant metabolic processes, chemotherapeutic drugs or temperature shifts, result in common morphological changes consisting of the formation and shedding of membrane vesicles from the injured cell surfaces, i.e., apoptosis. This dynamic cell surface membrane behavior appears to be dependent on the disruption of cytoplasmic microtubules. Concomitant with the altered cell surface morphology, certain physiological and biochemical events have been found to be associated with cell injury. These include changes in membrane permeability, elevated oxygen consumption rates and nuclear DNA fragmentation. However, it remains to be experimentally established which of these biological changes defines a state of irreparable cell injury and/or programmed cell death (PCD).

Selective cell injury and death is the goal of many therapeutic modalities aimed at the destruction of malignant cells. On the other hand, prevention of cell injury is desirable in autoimmune diseases such as systemic lupus erythematosus, thyroiditis, insulin dependent diabetes and many others. Injury to the vascular endothelium may play a role not only in thrombosis, atherosclerosis and hypertension, but may also provide the avenues for the metastasis of malignant cells.

The objective of the present review is to compare and evaluate the cell injury process induced by effector lymphocytes with that caused by low temperature. The latter mimics most, if not all, the currently known criteria of immune effector cell mediated PCD of target tumor cells.

Key Words: Cell injury, ion channels, Zn^{++}, Ca^{++}, signal transduction, hyperthermia, lipid metabolism, biosomes.

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Immune Effector Cell Induced Cell Injury

Morphologic criteria for cell injury

The major advances made in the understanding of the mechanism by which immune effector cells injure or trigger programmed cell death (PCD) of tumor cells, with eventual lysis, have been derived from the ^{51}Cr release assay developed by Brunner *et al.* (1968). This assay is based on the finding that live tumor cells incorporate $Na_2\ ^{51}CrO_4$ and release it, in a reduced form, when these cells are exposed to cytolytic lymphocytes. The percentage of specific ^{51}Cr released by the tumor cells was shown to correlate with their progressive loss of clonability after various time intervals of contact with effector lymphocytes.

When the effector-target cell conjugates are examined in the scanning electron microscope, microvillar extensions of the tumor cell surface membrane are observed over the apposing effector lymphocyte (Fig. 1) followed by blebbing of the entire tumor cell surface (Fig. 2). These blebs lead to the formation of closed membrane vesicles (MV) which detach from the tumor cell surface and tend to adhere preferentially to the apposing effector lymphocyte (Liepins *et al.*, 1978). Studies with isolated MV have showed them to be immunogenic in mice syngeneic to the tumor, thus indicating that they bear tumor associated antigens (TAA; Liepins, unpublished data). In some instances, the MV form a barrier between lymphocytes and target cells, suggesting a possible tumor escape mechanism (Fig. 3). The frequency of effector-target cell conjugates with altered tumor cell morphology precedes but correlates with the kinetics of specific ^{51}Cr release (Liepins *et al.*, 1978). Late in the ^{51}Cr release period, target cells with a spongy appearance suggestive of membrane perforations analogous to those caused by antibodies and complement, could be detected (Fig. 4).

The effector T-cell lytic agents are currently thought to be contained in cytoplasmic granules, i.e, lysosomes which may be released by local exocytosis at the effector-target cell junctions. These granules contain lytic enzymes (cytolysins) and polyperforins which are

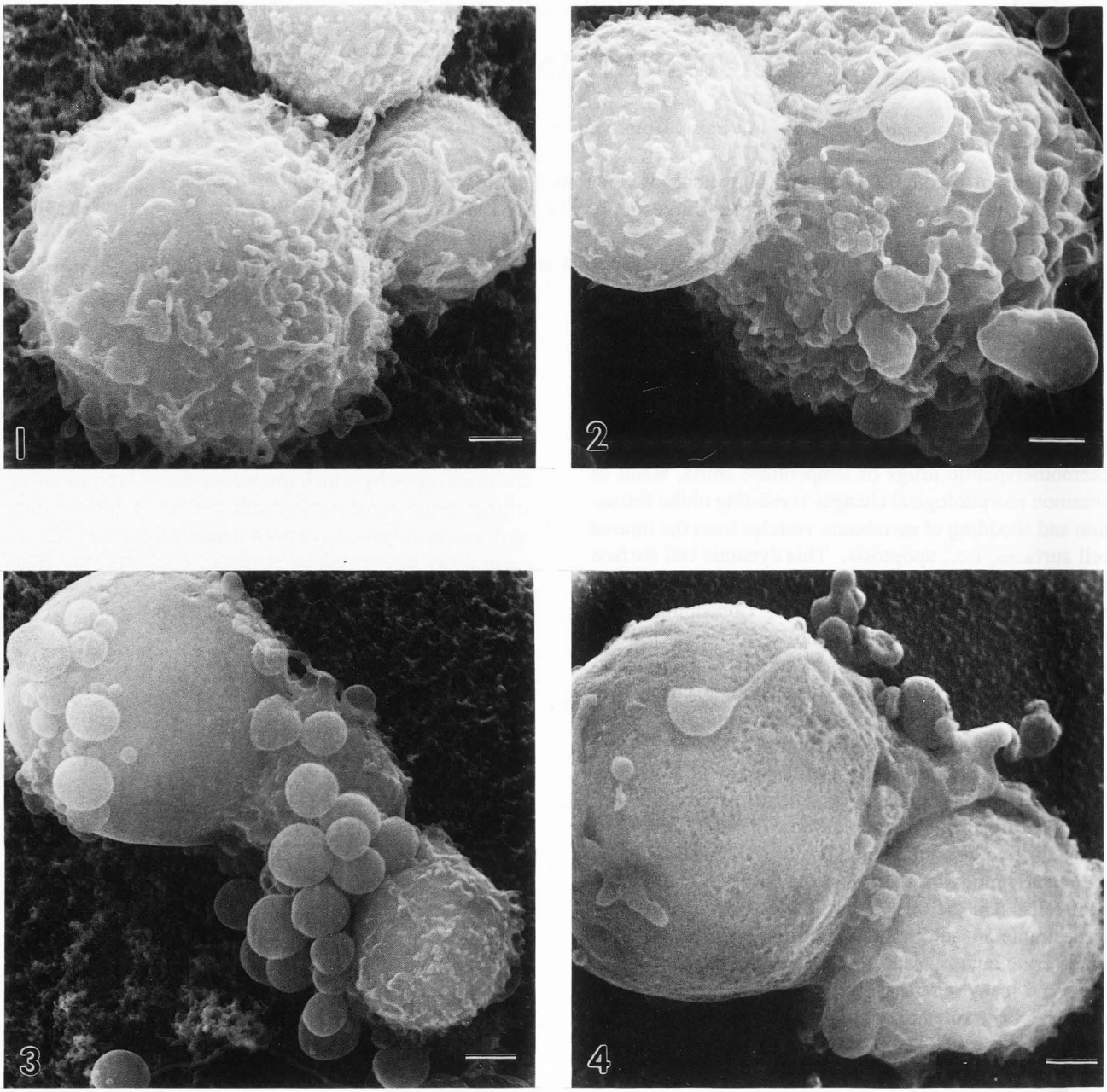
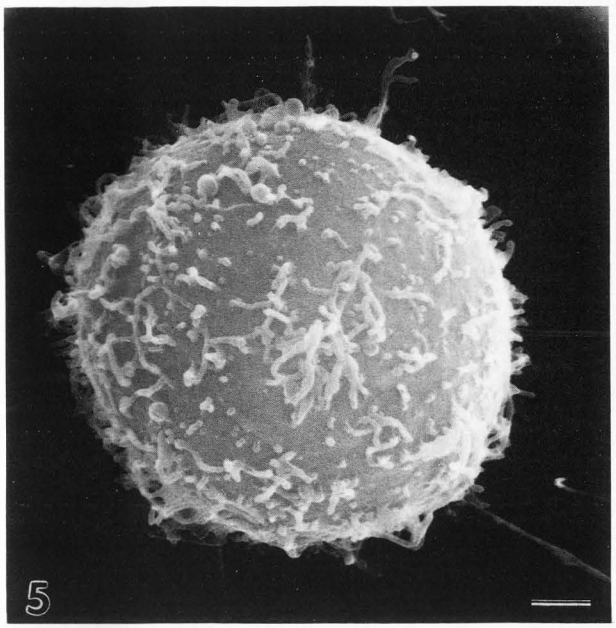


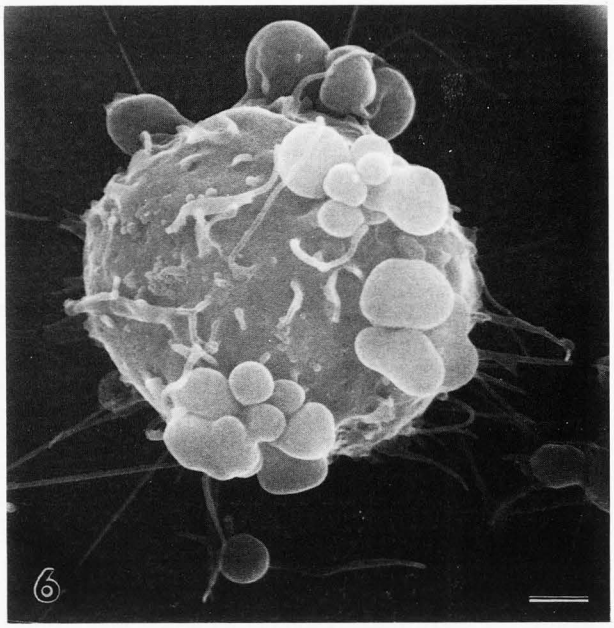
Figure 1. Alloimmune effector T-lymphocytes (small cells) in contact with a mastocytoma P815 tumor target cell. Microvilli of the tumor cell extend over the surface of one of the apposing T-cells. Bar = 1 μm.

Figure 2. Blebbing or vesiculation of the target tumor cell surface occurs shortly after contact with immune T-cells has been established. This tumor cell surface behavior represents the most frequently occurring event associated with the cell injury process. Bar = 1 μm.

Figure 3. Blebbing of the tumor cell surface leads to the formation and shedding of membrane vesicles which attach preferentially to effector T-cells. This vesicle behaviour suggests that these tumor cell products display antigenic properties, i.e., tumor associated antigens, as those of the parent tumor cells and may thus contribute to a tumor escape mechanism by blocking effector-target cell conjugate formation. Bar = 1 μm.

Figure 4. Apparent porous lesions in the tumor cell surface membrane appear during the later stages of the ^{51}Cr release assay. Effector lymphocytes remain attached to the tumor cells throughout the entire sequence of morphological events described. Bar = 1 μm.





Figures 5 and 6. Low temperature shift (0°C to 37°C) induced cell surface morphology changes. Control lymphocytic leukemia (L1210) cell before low temperature treatment (**Figure 5**). Blebbing and shedding of membrane vesicles occurs when these cells are submitted to the low temperature shift of 0°C/1 hr to 37°C/1 hr (**Figure 6**). Bar = 1 μm.

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thought to insert in the target cell membrane during the lytic process (Dennert and Podack, 1983). Alternatively, proteases concealed within the cell surface membrane of effector T-cells and natural killer cells have been implicated in the early stages of the target cell injury process (Lavie *et al.*, 1985).

Blebbing of the target cell surface has been consistently found to occur during T-cell mediated injury of tumor cells (Liepins *et al.*, 1978; Sanderson, 1981), as well as with other forms of cellular injury, e.g., chemotherapeutic drugs (see transmission electron micrographs in **Discussion with Reviewers**) and anoxia (Trump *et al.*, 1979). This has led to the notion that this cell surface behavior is a manifestation of irreversible cell injury which leads to cell death (Lemastèrs *et al.*, 1987). The criteria for cell death appear to be highly subjective, meaning different things to different investigators, i.e., loss of clonability, prolonged cell division cycle, membrane permeability changes to ^{51}Cr and trypan blue, blebbing of the cell surface (apoptosis or zeiosis), chromatin condensation [as demonstrated by transmission electron microscopy (TEM) studies], DNA fragmentation, etc. Lysis or cytolysis is an inferred event based primarily on changes in target cell membrane permeability to ^{51}Cr and/or trypan blue. A definite criteria for either lethal cell injury or cell death has yet to be established (Wolman, 1985).

Physiological and biochemical parameters associated with immune effector cell induced programmed cell death

The process of immune cell mediated injury requires metabolic energy, Mg^{++} ions for the formation of the effector-target cell conjugates and Ca^{++} ions for the process to occur as measured by changes of the target tumor cell membrane permeability to ^{51}Cr (Henney, 1973). The inherent difficulty of a two cell system in assigning the energy and/or ionic requirements to either of the interacting cells has made interpretations of these and other data difficult. For example, the T-cell mediated injury process of tumor cells requires oxygen, but the level at which it is required, i.e., for the effector or the target cell, is not known (Nathan *et al.*, 1982). More recently, exceptions for the Ca^{++} requirement in immune cell mediated injury process have been reported (Tirosh and Berke, 1985).

From the biochemical standpoint, it has been found that immune effector cells induce target cell nuclear DNA fragmentation (Duke *et al.*, 1983). Agarose gel electrophoresis of the cell nuclear DNA showed that the DNA-fragments consisted of discrete multiples of about 200 base-pair sub-units suggesting the involvement of an endonuclease in the T-cell mediated lytic process. The DNA fragmentation process was Zn^{++} sensitive and independent of protein and RNA synthesis (Duke *et al.*,

1983; Russell and Dobos, 1980). It should be noted, however, that Zn^{++}, at half the concentrations used in the DNA fragmentation studies mentioned, has been shown to stabilize cytoplasmic microtubules, the cell surface membrane morphology and to inhibit calmodulin function (Maro and Bornens, 1979). As reported by Bereza *et al.* (1982) a variety of antihemolytic drugs are also inhibitors of calmodulin function and act as membrane stabilizing agents (as measured by protection from osmotic lysis). Thus, Zn^{++}, a known calmodulin inhibitor, may protect cells from PCD by inactivating cell surface membrane associated calmodulin and not necessarily at an endonuclease level. Furthermore, the patch-clamp studies of Bustamante (1993) have demonstrated that micromolar concentrations of Zn^{++} blocked ion flow through the nuclear pore complex (NPC). Since ion conductance through the NPCs, as measured by this sensitive technique, is only possible when these structures are not transporting signal molecules, i.e., transcription factors, these findings indicate that Zn^{++} blocks ion flow through these functionally and structurally complex nuclear envelope components. Thus, this evidence indicates that Zn^{++} may function at the NPC level and not on the putative Zn^{++} sensitive endonuclease. The ability of Zn^{++} ions to modulate the NPCs open-close states, is not a unique property of the NPCs, but seems to be a property of proteins with specific amino acid sequences (Walker *et al.*, 1994).

The Low Temperature Induced Cell Injury Model

We have reported that perturbation of the cytoskeletal system by drugs or temperature shifts was sufficient to induce the formation and shedding of membrane vesicles (MV) from tumor cell surfaces (Figs. 5 and 6) (Liepins and de Harven, 1982; Liepins and Hillman, 1981; Liepins and Younghusband, 1985). The process of formation and shedding of MV induced by drugs or temperature shifts, was morphologically analogous to that occurring during the immune effector cell mediated injury and apoptosis of tumor cells (Liepins, 1982; Liepins *et al.*, 1978).

Tumor cells, when incubated at the microtubule disruptive temperature of 0°C and shifted to 22°C, form and shed MV without changes in cell surface membrane permeability. However, if cells were subjected to a temperature shift of 0°C to 37°C, the MV shedding process was associated with changes in cell membrane permeability to ^{51}Cr. These morphology and membrane permeability changes are analogous to those induced by immune effector cells, i.e., apoptosis or PCD. The microtubule stabilizing agent, deuterium oxide (D_2O), effectively inhibited both the low-temperature induced MV shedding process and the membrane permeability changes which occurred in the 0°C to 37°C temperature shift (Liepins, 1983). Furthermore, this microtubule stabilizing agent was also found to inhibit, in a dose dependent manner, the T-lymphocyte mediated injury of tumor target cells (Liepins, 1983). Thus, target cell microtubule disruption appears to be necessary for both the MV shedding process and the membrane permeability changes induced either by low temperature shifts or effector T-lymphocytes.

We have also investigated the effects of low temperature shifts on cell nuclear DNA integrity. These studies showed that when the MV shedding process was induced by the 0°C to 22°C temperature shift, nuclear DNA fragmentation did occur, but the DNA fragments remained within the cell. However, in the 0°C to 37°C temperature shift, DNA fragmentation occurred more rapidly and the cell surface membrane became permeable to the DNA fragments (Fig. 7) (Liepins and Younghusband, 1985). Thus, in the latter condition, the low temperature shift induced cellular changes which mimicked the T-cell mediated PCD process in all major parameters: cell surface morphology changes (as manifested by formation and shedding of MV); changes in membrane permeability to ^{51}Cr, as well as DNA fragmentation and loss across the cell surface membrane.

The use of low temperature for the handling of cells in tissue culture laboratories has become an insiduous procedure which may introduce an inadvertent temperature shifts from 0°C to 22°C or 0°C to 37°C. Such temperature shifts may result not only in cell injury as described above, but have also been shown to induce the selective loss of cell surface receptors (Kaplan and Keough, 1982), as well as altering values of lateral diffusion coefficients of hormone receptors and/or antigens (McCloskey and Poo, 1984). Current evidence indicates that the anchorage and distribution of cell surface molecules is not only dependent on the physico-chemical properties of the cell surface membrane, but also on a highly organized cytoskeletal system of filaments and microtubules which are sensitive to temperature shifts (Liepins, 1982; Liepins and Hillman, 1981), pharmacologic agents (inter alia, chemotherapeutic and antipsychotic drugs), ionophores, calmodulin inhibitors, cytochalasins, ions (Ca^{++}, Mg^{++}, Na^{+}, K^{+}, Zn^{++}), interferons, etc. (Aszalos *et al.*, 1985; Bourguignon and Bourguignon, 1984).

Role of Secondary Intracellular Messengers in Cell Injury: Ca^{++}, Diacylglycerol and Inositol 1,4,5 Triphosphate

Although most cells contain a great deal of Ca^{++}, most of it is not free in the cytoplasm but is predominantly bound to proteins, such as calmodulin, as well as

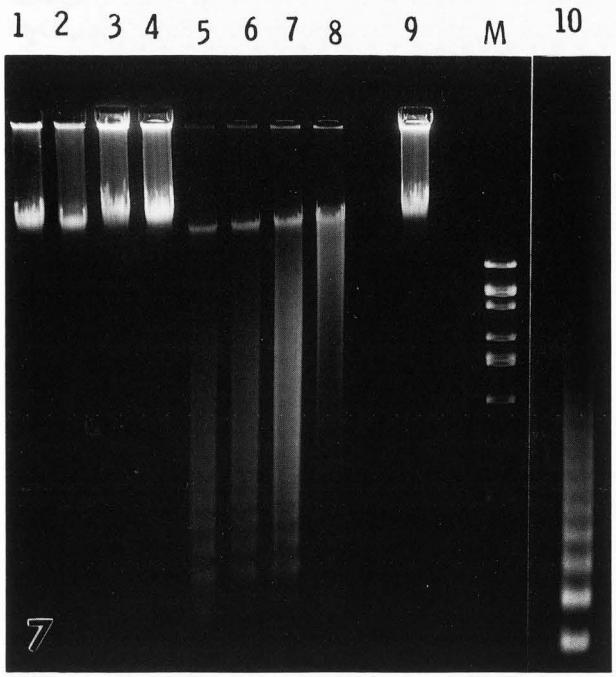

Figure 7. Nuclear DNA fragmentation in temperature shifted cells analyzed by agarose gel electrophoresis: Lanes 5 and 6 represent DNA fragmentation that occurs in P815 cells exposed to 0°C for one hour and shifted to 37°C for 1 hour and 2 hours respectively; lanes 7 and 8 represent DNA fragmentation in cells exposed to 0°C/1 hr and shifted to 22°C for 2 hours and 1 hour respectively. Lanes 1, 2, 3 and 4 represent DNA from cells exposed to 0°C/1 hr in the presence of tissue culture medium containing 90% D_2O, and shifted to 22°C and 37°C, showed no fragmentation of their DNA. Lane 9: DNA extracted from control cells not subjected to low temperature (0°C). Lane M: molecular weight markers ranging from 8,000 down to 1,000 base pairs. Lane 10: represents DNA recovered from tissue culture medium from cells shifted from 0°C/1 hr to 37°C. The fragmented DNA of cells shifted from 0°C to 22°C remained intracellular.

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sequestered in mitochondria and the endoplasmic reticulum (Hochachka, 1986; May *et al.*, 1985). Furthermore, several ATP driven pumps and ion exchange systems remove free Ca^{++} from the cytoplasm if its concentration begins to rise. All of these factors maintain a relatively constant intracellular free Ca^{++} concentration of about 10^{-9}M (May *et al.*, 1985). Since the Ca^{++} concentration in the extracellular environment is in the mM range, there is a concentration gradient of four or more orders of magnitude across the cell membrane. Transient increases in free cytosolic Ca^{++} are thought to act as intracellular signals, i.e., second messenger functions. Cytosolic increase of Ca^{++} can be accomplished by a transient opening of the cell surface membrane Ca^{++} channels, thus allowing movement of Ca^{++} down an electrochemical gradient. Some cells have Ca^{++} channels that can be opened by the action of an agonist on a receptor or by changes in membrane potential, i.e., gating. Alternatively, Ca^{++} can be released from internal storage sites by hormones, amino acids and neurotransmitter interactions with specific cell surface receptors which results in the breakdown of phosphatidyl-inositol biphosphate (PIP_2) producing diacylglycerol (DG) and inositol 1,4,5,-triphosphate (IP_3). The latter interacts with intracellular receptors leading to the release of Ca^{++} from the intracellular storage sites, whereas DG, in conjunction with Ca^{++} activates protein kinase C (PKC) (May *et al.*, 1985).

Several lines of evidence indicate that PKC modulates cell surface membrane ion conductance by phosphorylating ion channel proteins, ion pumps and ion exchange proteins (Levitan, 1985). Furthermore, it has been proposed that PKC plays a role in the extrusion of cytosolic free Ca^{++}, and that a Ca^{++} transport ATPase is a possible target for this protein kinase. Clearly, Ca^{++} plays multiple roles in the homeostasis of cellular functions and its free cytosolic concentration is precisely regulated by membrane transport systems, binding to proteins and by sequestration in intracellular sites (Kuno and Gardner, 1987).

Target cell surface blebbing is an early event in T-lymphocyte mediated injury of tumor cells (Liepins *et al.*, 1978), as well as in hypoxic and toxic injury of cells (Lemasters *et al.*, 1987). As pointed out by Lemasters *et al.* (1987), a rise in cytosolic free Ca^{++} has been suggested as the stimulus for cell surface bleb formation and the final common pathway to irreversible cell injury: PCD. These investigators evaluated the often implied role of Ca^{++} in the cell injury process by deploying digitized low-light video microscopy of fura-2 to correlate the blebbing process with the level of cytosolic free Ca^{++}. Within minutes after addition of cyanide and iodoacetate to induce hypoxia, blebbing occurred, and by 30 minutes more than 70% of the cells displayed surface blebbing. During the bleb formation period, the level of cytosolic free Ca^{++} remained unchanged. In most cells, one or more cell surface blebs formed as the chemically induced hypoxia progressed. Finally, some blebs ruptured or were reabsorbed with concomitant loss of Ca^{++}, i.e., fura-2 fluorescence (Lemasters *et al.*, 1987). These events were considered by Lemasters *et al.* (1987) to represent the abrupt transition from reversible to irreversible injury. Furthermore, upward and downward modulation of cytosolic Ca^{++} by hormones (phenylephrine, vasopressin and epidermal growth factor), Ca^{++} ionophore (ionomycin) and the Ca^{++}

chelator EGTA, were all measurable in terms of fura-2 fluorescence. This upward and downward modulation of cytosolic free Ca^{++} did not result in blebbing nor in the loss of viability, i.e., propidium iodide exclusion from cells (Lemasters *et al.*, 1987). These data shed doubt on the role of changes in free cytosolic Ca^{++} concentrations in the manifestation or consequence of cell injury. Thus, the long-standing notion that elevated cytosolic Ca^{++} levels lead to cell injury and PCD or apoptosis, may need to be reevaluated. However, Ca^{++} may play a role in the cell injury process by activation of Ca^{++} binding proteins such as calmodulin with subsequent activation of phospholipases A and C, enzymes required for initiation of the arachidonic acid cascade and the catabolism of membrane phosphatidyl-inositolbiphosphate (PIP_2), respectively (Kuno and Gardner, 1987).

Lipid Metabolism and Cell Injury

Low temperature shifts (0°C/15 min to 37°C/10-30 min), analogous to the ones used in this laboratory (Liepins and Hillman, 1981; Liepins and Younghusband, 1985), have been shown by Pryme (1985) to stimulate the incorporation of choline in intracellular membrane phospholipids followed by a rapid flow through the endomembrane system. Furthermore, cytotoxic effector cell mediated PCD may proceed via phospholipase activation resulting in the conversion of phosphalidylcholine (lecithin) to lysophosphatidylcholine (lysolecithin). The latter is a strong detergent and potent cytolytic agent which may cause lethal damage to the target cells (Fry and Friou, 1975; Liepins and Jayasinghe, 1985). Changes in lipid metabolism, due to phospholipase A activity has been reported to occur in target cells (Koren *et al.*, 1971) as well as in effector cells during the lytic process (Deem *et al.*, 1987).

There appears to be ample evidence for changes in phospholipid metabolism during cell injury, but the involvement of reactive oxygen intermediates seems unlikely because D_2O, which is known to prolong their half-life many fold (Kalyanaraman *et al.*, 1987), prevents (instead of enhancing) both the T-cell and the low temperature induced injury processes (Liepins, 1983; Liepins and Younghusband, 1985). Furthermore, we have found that D_2O reduces the elevated oxygen consumption rates, which occur during the MV shedding process of apoptosis, down to those of control non-shedding cells (Liepins and Jayasinghe, 1985). Thus, the high oxygen uptake rates associated with the low temperature induced cell injury process are more likely to be involved in the production of high energy metabolites, such as ATP, which may be required for the highly dynamic MV shedding process (Liepins and Hillman, 1981). D_2O did not alter the oxygen uptake rates of control cells which were not subjected to the 0°C/1 hr treatment ((Liepins and Jayasinghe, 1985). Thus, D_2O is unique in its biological affects in that it protects tumor cells from the low temperature as well as from T-cell mediated PCD process (Liepins, 1983; Liepins and Jayasinghe, 1985), as measured by changes in membrane permeability to ^{51}Cr and/or trypan blue, blebbing and shedding of MV from the cell surfaces and nuclear DNA fragmentation (Liepins and Younghusband, 1985).

Possible Role of Microtubules in Cell Injury

Since the major known biological effect of D_2O is the stabilization of cytoplasmic microtubules (Oslund *et al.*, 1980) from disruption by low temperature (Oliver and Berlin, 1982), it is likely that their integrity is necessary for the maintenance of normal cell morphology as well as function and possibly viability. The concept that microtubules may interact with and influence the properties of the cell surface membrane has been the focus of numerous studies (e.g., see reviews: Oslund *et al.*, 1980; Weber and Osborn, 1979). Weber and Osborn (1979) have suggested that microtubules interact with the cell surface membrane to maintain a uniform surface tension, which if disrupted may result in the outpocketing of a membrane region or domain, i.e., blebbing. This postulate is consistent with evidence demonstrating that drug- or low-temperature-induced disruption of microtubules occur initially at the cell periphery near the cell surface membrane coinciding with blebbing regions of the cell surface. Furthermore, microtubules have been reported to be associated with the cell surface membrane (Oliver and Berlin. 1982) and may be involved in the anchorage and shedding of cell surface receptors (Kaplan and Keough, 1982). Since the low temperature condition we have used for induction of apoptosis and nuclear DNA fragmentation process, is known to depolymerize microtubules, suggests that these structures play a role not only at the cell surface level but may also be involved, in a yet unknown manner, in protecting nuclear DNA from fragmentation (Liepins and Younghusband, 1985). Alternatively, D_2O may inhibit the zinc-sensitive endonuclease activity believed to be involved in the DNA fragmentation process (Duke *et al.*, 1983; Russell and Dobos, 1980) as well as in the gating of ion channels of the NPCs (Schauf and Bullock. 1980). Interestingly, antibody complement-mediated lysis of target cells, which is reported not to result in DNA fragmentation (Russell and Dobos, 1980), was found to be insensitive to D_2O (Liepins, unpublished).

Finally, it is worth noting that shedding of cell surface membranes via MV is not an event limited to *in vitro* situations but has been reported to occur *in vivo* in tumor bearing hosts (Alexander, 1974; Doljanski and

Kapeller, 1976; Taylor and Black, 1985), in hyperplastic thyroid epithelium (Zeligs and Wollman, 1977), during normal thymocyte differentiation (Roozemond and Urli, 1981) as well as in megakaryocyte production of platelets (Djaldetti *et al.*, 1979; Haller and Radley, 1983; Zucker-Franklin and Petursson, 1984) and in embryogenesis (Duvall and Wyllie, 1986). According to Wenner *et al.* (1985), apoptosis or blebbing appears to be closely associated with regulation of DNAase I endonuclease and involves new protein synthesis. These investigators suggest that tumor promoters affect covalent modifications, i.e., phosphorylation of the endonuclease target sites or perturbation of cytoskeletal components, e.g., monomeric actin which is an inhibitor of DNAase I, resulting in a direct modulation of endonuclease action. Each of these possibilities can arise from membrane-associated reactions. Non-repair associated DNA fragmentation may result in decreased cell survival and tumor promoters such as phorbol esters and indole alkaloids may inhibit a generalized breakdown of the genome sufficiently to allow replication of even a small number of affected cells. Thus, the retention of cells which contain an inheritable transformed genome can lead to a stable, clonogenic population of cells with transformed phenotype. Consequently, programmed cell death during embryogenesis is thought to represent a vulnerable time during which tumor promoters may exert their tumor initiation reactions (Wenner *et al.*, 1985).

The Role of K^+ Channels in Cell Injury

Ion channels are integral membrane proteins through which ions can passively flow down their electrochemical gradient at rates exceeding 10^6 ions/sec (Gallin, 1986; Lecar, 1985). Shifts in the resting potential of cell-surface membranes may result from the activation of specific ion channels which are thought to be involved in a variety of cellular functions including nerve conduction, muscle-cell contraction and relaxation, cell proliferation, protein synthesis (DeCoursey *et al.*, 1984; Deutsch *et al.*, 1986; Lecar, 1985; Meldolesi and Pozzan, 1987; Panet *et al.*, 1985) as well as in processes associated with cell PCD (Liepins and Younghusband. 1987; Schlichter *et al.*, 1986; Trump *et al.*1979). Most of the ion channels can exist in either an open or a closed state and the transition between these states, i.e. gating, is regulated by the voltage across the membrane or by the binding of a ligand to a specific receptor associated with the channel molecule (Meldolesi and Pozzan. 1987). In some cells the protein molecules constituting the ion channels, or possibly components very close to the channel molecule, e.g., cytoskeletal structures, have been found to be phosphorylated in response to a variety of physiological conditions leading to changes in membrane ion permeabilities and thus function (Levitan, 1985).

As pointed out by Hochachka (1986), most mammalian cells are cold-sensitive and lose intracellular K^+ when subjected to low temperatures. The K^+ efflux at low temperatures is apparently greater than the energy-dependent inward pumping, resulting in a net loss of intracellular K^+ as well as an imbalance in other ions such as Na^+, Ca^{++} and Mg^{++}. On the other hand, cold-tolerant cells during hypothermia are able to maintain near-normal membrane functions, i.e., they maintain relatively normal ion concentration differences between various internal and external compartments despite greatly reduced metabolic rates (Hochachka, 1986).

K^+ channel activity has been implicated in T-lymphocyte activation by phytohemagglutinin (PHA), allogeneic mixed lymphocyte responses, protein, DNA and interleukin 2 (IL2) synthesis (Chandy *et al.*, 1985). These lymphocyte functions were abrogated in a dose dependent manner, by the classical K^+ channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP) (Chandy *et al.*, 1984). Moreover, K^+ channel activity has been reported to be involved in the delivery of the "lethal hit" by cytotoxic T-lymphocytes (Farber *et al.*, 1981; Fukushima *et al.*, 1984) and human NK cells (Schlichter *et al.*, 1986). Other studies using target cells loaded with ^{86}Rb, have suggested the presence and function of K^+ channels in tumor cells undergoing T-cell mediated lysis (Ferluga and Allison, 1974; Henney, 1973; Martz, 1976; Sanderson, 1981). Thus, K^+ channels have been implicated in the killing process both at the effector as well as at the target cell level. In a two cell type system, as that of effector plus target cells, it is difficult to demonstrate by pharmacological means that a particular ion channel function is a requirement for effector cells to deliver their cytolytic effects or for the target cells to initiate the suicidal biochemical pathways involved in the PCD process leading to death.

Using the low temperature shift induced cell injury process, we have investigated whether K^+ ion channel function may be also required for tumor cell-surface vesicle formation and shedding, changes in membrane permeability, and nuclear DNA fragmentation, i.e, PCD. Results from these studies demonstrated that the two classical K^+ channel blockers TEA and 4-AP inhibited, in a dose dependent manner, the MV formation and shedding process as well as nuclear DNA fragmentation and membrane permeability changes (Liepins and Younghusband, 1985, 1987). Furthermore, scanning electron microscopy revealed that the low-temperature shift induced MV formation and shedding was indistinguishable from that occurring during alloimmune T-lymphocyte-mediated injury of tumor cells (Liepins and Younghusband, 1987; Liepins *et al.*, 1978). These re-

sults clearly indicate that function of K^+ channels are required for tumor cell susceptibility to the low temperature induced PCD process (Liepins and Younghusband, 1987). Thus, K^+ channel activity appears to be required for both the triggering or delivery of lytic signals from the effector cells as well as for the target tumor cells to initiate the self-destructive PCD process. It seems likely that different types of K^+ channels may be involved at the effector and target cell level producing drastically different cellular events. Further voltage-clamp studies in conjunction with channel blockers, specific for the various types of K^+ channels, will be required to clarify their apparent dichonomous roles in effector and target-tumor cells, respectively.

The Role of Membrane Vesicles (MV) in Disease

Hanker and Giammara (1983) have reported that neutrophils from patients with acute infection or myeloid leukemia, formed and shed platelet-size MV which contained myeloperoxidase, acid hydrolases and occasionally nuclear fragments. Since these MV were present in bacteria laden pus and inflammatory exudates, and their ingestion by macrophages, these structures were thought to be involved in the immune response to pathogens or to contribute to trauma and healing by facilitating the deployment of neutrophil functions via these cell products. On the other hand, neutrophils from healthy individuals, when stimulated to produce reactive oxygen intermediates, have been reported to induce malignant transformation of normal murine fibroblasts which produced tumors when injected into nude mice (Weitzman, *et al.*, 1983). The manner by which activated human neutrophils induced malignant transformation was not identified. However, the demonstration of myeloperoxidase activity in the platelet-size MV produced by neutrophils (Hanker and Giammara, 1983) raises the possibility that these vesicles may serve as vectors for the induction of carcinogenesis in normal cells.

It has been recently demonstrated by Dang *et al.* (1985) that fibrinogen fragments of 78,000 and 94,000 dalton molecular weights caused profound disorganization and injury of cultured bovine aortic endothelial cells, consisting of cell retraction, cell rounding and the formation of prominent cell surface protrusions, i.e., blebs, with concomitant disorganization of actin microfilaments. Such cellular changes are also characteristic of cellular injury caused by toxic chemicals (Jewell *et al.*, 1982), immune T-cells (Liepins *et al.*, 1978), neutrophils during acute infection (Hanker and Giammara, 1983) and possibly by reactive oxygen intermediates (Guthrie *et al.*, 1984).

The production of reactive oxygen intermediates by human mononuclear cells, stimulated by small amounts of bacterial endotoxin (Guthrie *et al.*, 1984) suggests that stimulated neutrophils could be involved in direct endothelial cell damage. Alternatively, plasminogen activator enzyme released by mononuclear cells could generate the active fibrinogen fragments that cause endothelial cell injury (Dang *et al.*, 1985). Furthermore, the plasma levels of fibrinogen fragments have been found to be elevated in patients with acute respiratory distress syndrome (Rinaldo and Rogers, 1982), thrombotic thrombocytopenia purpura (Ridolfi and Bell, 1981) and disseminated intravascular coagulopathy (Bell, 1980). These syndromes share the salient features of elevated fibrinogen-fibrin degradation products and severe endothelial cell abnormalities (Dang *et al.*, 1985). The injured vascular endothelium may also serve as avenues for tumor metastatic spread.

Taylor and Black (1985) have reported that MV produced by metastatic melanoma cell lines inhibit antigen presentation by macrophages, via enhancement of prostaglandin synthesis, resulting in immune non-responsiveness. As pointed out by these investigators, tumor derived MV have been implicated in the release of plasminogen activator, platelet aggregation factors which facilitate tumor emboli formation, inhibition of macrophage cytotoxicity, competitive binding of antitumor antibodies and immunosuppression by activation of T-suppressor lymphocytes. On the other hand, studies in our laboratory have shown that membrane vesicles produced by the low temperature shift method to be immunogenic, in that mice immunized with MV derived from P815 tumor cells rejected repeated lethal doses of syngeneic P815 tumor cell challenges. Disruption of the MV by homogenization abrogated their immunogenicity. These results indicate that induction of tumor immunity requires the presentation of antigens in a proper configuration (Liepins, unpublished).

In view of the fact that right-side-out MV derived from a variety of cells, growing both *in vivo* as well as *in vitro*, have unique biological functions, it is proposed that such MV be referred to as "biosomes" to distinguish them from vesicles produced by cell disruption methods, i.e., microsomes, or by biochemical construction methods, i.e., liposomes. The latter have been utilized by Goldstein and Mescher (1987) to investigate the requirements of antigen density and configuration necessary to elicit an effective immune response. These artificial membrane vesicles have been termed pseudocytes (artificial cells) by Goldstein and Mescher (1987). It is evident that the understanding of the cell injury (PCD and/or apoptosis) and the membrane vesicle shedding process, in precise biochemical terms, could contribute significantly to the development of therapeutic modalities targeted at a particular aberrant biochemical pathway.

Concluding Remarks

Despite the considerable efforts directed towards the understanding of cell injury, and by inference programmed cell death, these events are experimentally unresolved and remain poorly defined. The tenacity with which malignant cells recover and/or escape from highly toxic chemotherapeutic drugs as well as from the host immune defense mechanisms has yet to be defined in biochemical terms. Historically, the study of biological processes associated with preservation of life and not death, has occupied the center stage of biological and clinical research. It is only recently that some concerted research efforts have been directed towards the understanding of the biological events believed to be associated with lethal cell injury. What has emerged from this work is that cell injury, and implicitly programmed cell death, is the result of an orderly process with characteristic morphological (blebbing and shedding of cell surface membranes), physiological (membrane permeability changes to ions and elevated oxygen uptakes) and biochemical (nuclear DNA fragmentation) manifestations. The finding that extensive nuclear DNA fragmentation occurs during immune lymphocyte mediated injury of tumor cells as well as in cells submitted to low temperature shifts, offers the most tenable biochemical criteria defining a stage of cell injury which may be lethal, i.e., point of no return. This definition is contingent upon future studies demonstrating that the nuclear DNA fragmentation is irreparable.

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Discussion with Reviewers

K.M. Anderson: In some statements, it appears the authors equate shedding of membrane vesicles with apoptosis; in other statements a more conventional view of the process is presented!

Authors: Our *in vitro* low temperature shift (0°C/1 hr → 37°C) can be followed through the various stages of apoptosis or PCD. Without exception, tumor cell surface blebbing proceeds to the formation of free, right side out, membrane vesicles which can be isolated in a density gradient medium (Liepins and Hillman, 1981).

K.M. Anderson: There is a developing view that apoptosis and "programmed cell death" are not really identical processes; since this is an emerging view and not generally agreed upon, the point can be left in abeyance.

Authors: We do not think that such an important issue should be "left in abeyance". On the contrary, we think that this is a critical issue which should be experimentally addressed and vigorously debated. This important concept will be the focus of the forthcoming apoptosis sessions at the next Scanning Microscopy meeting, May 6-11, 1995 in Houston.

K.M. Anderson: Deuterium oxide is said to extend the half life of reactive oxygen, yet the reference (Kalyanaraman *et al.*, 1987) is to singlet oxygen, not a true free radical. Are other radicals involved?

Authors: We have not directly measured singlet oxygen levels nor free radicals in low temperature induced apoptosis, hence this issue remains to be experimentally resolved.

K.M. Anderson: Are elevated oxygen consumption rates at low temperature due to an increased synthesis of

ATP, I think that unlikely; or more likely, due to uncoupling of oxidative phosphyoylation? Normally metabolic rates decline with lowered temperature, a sort of inverse Q/10 rule.

Authors: The reviewer is correct in that oxygen and dependent metabolic rates drop at low temperature. It is during the shift in temperature back to physiological levels when elevated oxygen consumption rates are significantly higher than those of cells which were not subjected to low temperature (0°C/1 hr) treatment (cf. Liepins and Jayasinghe, 1985). Uncoupling of oxidative phosphorylation, as suggested by the reviewer, may be the underlying biochemical event responsible for high oxygen consumption rates.

I.D. Bowen: You state: "A definite criteria for either lethal cell injury or cell death has yet to be established (Wolman, 1985)". This statement is as true now as it was in 1985. Since the authors use the words "yet to be" inclusion of more recent references in this area, e.g., I.D. Bowen [Cell Biol. Int., 1993, **17**(4), 365-380], would strengthen their case.

Authors: You are quite correct, your recent review, bring into focus possible differences in morphology as well as biochemistry of cell death pathways. Thus, recent evidence indicates that different cells may choose different biochemical pathways leading to the common final event of death. Nevertheless, consensus of criteria for cell death modalities need to be established in order to emerge from the current chaos of terminology applied indiscriminately to a variety of experimental systems.

I.D. Bowen: DNA fragmentation is not a good diagnostic feature of PCD [Bursch W, Oberhammer F, Schulte-Hermann, R (1992) Cell death by apoptosis and its protective role against disease. TIPS **13**, 245-251; Zakeri ZF *et al.* (1993) Delayed ribonucleosomal DNA fragmentation in programmed cell death. FASEB J. **7**, 470-478] and can be spuriously engendered by Ca^{++}, etc. However, if the technique is being employed, a more rigorous regime involving 3'end labelling with ^{32}P (see Zakeri *et al.*, 1993, mentioned above) should have been adopted.

Authors: Recent evidence strongly supports this reviewer's comments, which are also substantiated in the recent review of I.D. Bowen cited in the previous question of Dr. Bowen. In fact, current unpublished data from our laboratory clearly show that the morphology of apoptosis, induced by alkaloids, is associated with increases in nuclear DNA content, i.e., polyploidy, instead of DNA fragmentation on loss from the cells.

E. Falcieri: Apoptosis is a complex mechanism, not so easy to define, as the authors repeatedly suggest. Nevertheless, the apoptotic cell shows very characteristic features which mostly appear only in this biological event. Chromatin margination and cup-shaped aggregations, as well as final micronuclei formation, are indeed typical aspects, never present in other phenomena implying deep nuclear perturbations. All these changes, which occur in the inner cell, can be preferably identified in sectioned specimens.

Authors: This is indeed an important point that we had neglected to mention since nuclear chromatin condensation perhaps remains as the only non-controversial criteria of apoptosis. It is worthy to site the recent work of Falcieri *et al.* (Nuclear Pores in Apoptotic Cells, Histochem. J. **26**, 754-763, 1994), where they show migration of nuclear pores towards diffuse chromatin areas, whereas dense chromatin areas appear in nuclear pore free areas. Figures 8-11 from our laboratory present transmission electron micrographs of low temperature induced apoptosis of P815 mastocytoma cells which illustrate nuclear changes in this experimental system.

E. Falcieri: In the text you state "MV tend to adhere preferentially to the opposing effector cell ...". It should be interesting to clarify, if known, the molecular basis for this mechanism.

Authors: We do indeed have data from our immunological studies with isolated MV shed by P815 cells, that these right side out vesicles bear tumor associated antigens (TAA) since they are immunogenic to the syngeneic host, i.e., DBA/2 mice. Moreover, when isolated MV are added to *in vitro* cell mediated cytolysis assays of P815 cells, they bind to effector lymphocytes and cause a delay in specific ^{51}Cr release kinetics, i.e., blocking of lymphocyte mediates lysis of target cells (unpublished data).

E. Falcieri: In addition to TEM, among the criteria for identification for apoptosis, flow cytometry evidentiates a subdiploid peak (due to DNA loss.....), corresponding to apoptotic cell population after ethanol treatment and propidium iodide staining.

Authors: This is quite correct for our low temperature induced apoptosis model system. However, as mentioned in our reply to Dr. Anderson above, we have found that certain alkaloids induce classical morphology of apoptosis which is associated with an increase, and not a decrease in nuclear DNA content (unpublished data).

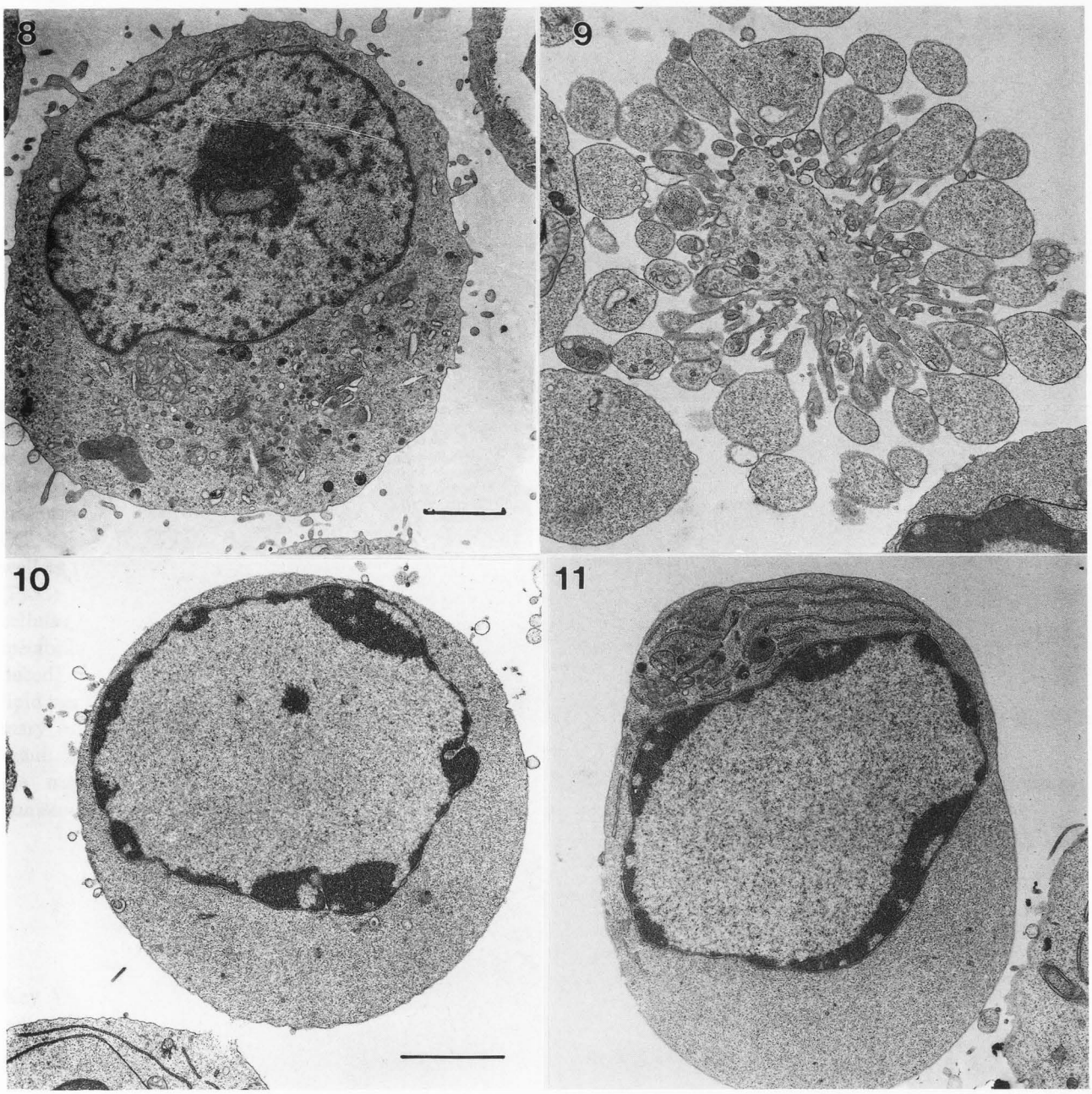

Figure 8. Transmission electron micrograph of a control P815 mastocytoma cell. Note the presence of cell surface microvilli, cytoplasmic organelles and euchromatic nucleus. Bar = 1 μm.

Figure 9. Tangential section through a P815 cell induced to form and shed membrane vesicles by the low temperature shift of 0°C/ 1 hr → 37°C/1 hr. Note that the blebs and/or membrane vesicles contain primarily RNP particles. Bar = 1 μm.

Figure 10. Section through a P815 mastocytoma cell after completing the low temperature induced membrane vesicle shedding process (apoptosis). Note the chromatin condensation along the nuclear envelope; cytoplasm devoid of organelles and the absence of cell surface membrane microvilli. Bar = 1 μm.

Figure 11. Different section plane of a P815 cell after completing the low temperature induced membrane vesicle shedding process. Note the limited amount of endoplasmic reticulum and cytoplasmic organelles. Note: cell size is significantly reduced by the membrane vesicle shedding process and not by cell shrinking as often claimed to be associated with apoptosis. Bar = 1 μm.