

# Constitutive Expression of the Machinery for Programmed Cell Death

M. Weil, M.D. Jacobson, H.S.R. Coles, T.J. Davies,\* R.L. Gardner,\* K.D. Raff, and M.C. Raff

Medical Research Council Developmental Neurobiology Programme, Medical Research Council Laboratory for Molecular Cell Biology and the Biology Department, University College London, London WC1E 6BT; and \*Imperial Cancer Research Fund, Developmental Biology Unit, Department of Zoology, Oxford OX1 3PS

**Abstract.** In the presence of cycloheximide (CHX) to inhibit protein synthesis, a high concentration of staurosporine (STS) induces almost all cells in explant cultures of 8/8 types of newborn mouse organs and 3/3 types of adult mouse organs to die with the characteristic features of apoptosis. Eggs and blastomeres also die in this way when treated with STS and CHX, although they are less sensitive to this treatment than trophectoderm or inner cell mass cells whose sensitivity resembles that of other developing cells. Human red blood

cells are exceptional in being completely resistant to treatment with STS and CHX. As (STS plus CHX)-induced cell deaths have been shown to display the characteristic features of programmed cell death (PCD), we conclude that all mammalian nucleated cells are capable of undergoing PCD and constitutively express all the proteins required to do so. It seems that the machinery for PCD is in place and ready to run, even though its activation often depends on new RNA and protein synthesis.

**M**ANY cells undergo programmed cell death (PCD)<sup>1</sup> during normal animal development (Glucksmann, 1951), and in most mammalian tissues PCD continues throughout life. Although the mechanism of PCD is still uncertain, there is increasing evidence that it has been conserved in evolution from nematode worms to humans and involves one or more members of the Ced-3/IL-1 $\beta$  converting enzyme (ICE) family of cysteine proteases (Yuan et al., 1993; for reviews see Thornberry et al., 1995, and Martin and Green, 1995).

When many types of mammalian cells are cultured in the absence of serum or extracellular signaling molecules, they undergo PCD (for review see Raff, 1992). Such findings have led to the proposal that all nucleated mammalian cells, except for blastomeres (Biggers et al., 1971), may require signals from other cells to avoid PCD (Raff, 1992). This death-by-default mechanism might ensure that a cell survives only when and where it is needed, just as the dependence on extracellular growth factors for cell proliferation is thought to ensure that a cell divides only when another cell is needed (Baserga, 1985). If extracellular signals are required to keep the death program suppressed,

then anything that blocks the intracellular signaling pathways activated by such survival factors should induce cells to undergo PCD. This was the rationale for our originally testing staurosporine (STS), a bacterial alkaloid that is a broad spectrum inhibitor of protein kinases (Tamaoki and Nakano, 1990), many of which are involved in intracellular signaling cascades. As expected, we (Jacobson et al., 1993, Ishizaki et al., 1993, Raff et al., 1993, Jacobson et al., 1994, Ishizaki et al., 1994, 1995) and others (Bertrand et al., 1994) have found that a high concentration of STS induces PCD in various cell lines and various types of dissociated primary cells in culture. It remains to be determined, however, whether it does so by blocking intracellular signaling pathways activated by extracellular survival signals.

Several lines of evidence indicate that the cell death induced by STS is bona fide PCD: (1) the morphology of the dead cells, analyzed by both light and electron microscopy, is typical apoptosis (Jacobson et al., 1993; Bertrand et al., 1994; Jacobson et al., 1994); (2) the sequence of organelle changes is characteristic of PCD, with nuclear changes, for example, occurring early and plasma membrane integrity maintained until late (Jacobson et al., 1994); (3) DNA degradation, which is thought to be an invariable feature of PCD, occurs (Bertrand et al., 1994); (4) overexpression of the *bcl-2* gene, which suppresses more physiological examples of PCD (for review see Reed, 1994), also suppresses STS-induced cell death (Jacobson et al., 1993, 1994); and (5) peptide inhibitors of the Ced-3/ICE family of cysteine proteases can suppress STS-induced PCD in a wide variety of cell types (see accompanying paper). Although the mechanism by which STS activates the death program is

Please address correspondence to M. Jacobson, MRC Laboratory for Molecular Cell Biology & Biology Department, University College London, London, WC1E 6BT, United Kingdom. Tel.: 44 171 380 7016. FAX: 44 171 380 7805.

1. *Abbreviations used in this paper:* CHX, cycloheximide; ICE, IL-1 $\beta$  converting enzyme; PCD, programmed cell death; PI, propidium iodide; STS, staurosporine.

unknown, this is also the case for almost all other inducers of PCD, so STS-induced PCD is not exceptional in this respect.

In principle, extracellular survival signals could be required to prevent either the production or the activation of proteins involved in executing the death program. The findings that inhibitors of RNA or protein synthesis can often suppress or delay PCD originally suggested that cells have to make new RNA and protein to undergo PCD and provided some of the most compelling original evidence that these deaths are suicides, in which the cells participate in their own demise (Tata, 1966; Duke and Cohen, 1986; Martin et al., 1988; Oppenheim et al., 1990). More recently, however, it has been shown that such inhibitors do not always suppress PCD and can even induce it (for review see Martin, 1993). STS-induced PCD in the cell lines and primary cells that have been tested, for example, is enhanced rather than suppressed by the protein synthesis inhibitor cycloheximide (CHX) and can even occur in the absence of a nucleus (Jacobson et al., 1994). Moreover, survival signals can inhibit PCD in the absence of a nucleus (Jacobson et al., 1994) or protein synthesis (Harrington et al., 1994). Such findings suggest that, in many cell types at least, the proteins required to execute the death program are constitutively expressed and extracellular survival signals act to prevent the activation rather than the production of these proteins.

Unlike most types of mammalian cells, blastomeres do not require extracellular signals to survive in culture: when cultured from 1-cell up to 16-cell mouse embryos, they can survive and divide in the absence of exogenous proteins or signaling molecules, even when cultured as isolated single cells (Biggers et al., 1971). It is unknown whether blastomeres are autonomous for survival because they can keep the death program suppressed in the absence of extracellular survival signals or because they lack a functional death program.

Here we show that, in the presence of CHX, a high concentration of STS induces PCD in almost all cells in explant cultures of all of the rodent organs we have tested, including newborn kidney, lung, muscle, pancreas, liver and skin, and adult kidney, lung, and pinna (the extracranial part of the ear). We also show that although mouse blastomeres and eggs are relatively resistant to STS-induced PCD compared to other developing cells, they too undergo PCD when treated with a very high concentration of STS, in the presence or absence of CHX; when blastomeres develop into inner cell mass (ICM) and trophectoderm cells, however, they acquire the sensitivity to STS-induced PCD that is characteristic of other developing cells. Human red blood cells, by contrast, which lack a nucleus and other organelles, are impervious to STS and CHX treatment. These findings suggest that, with the exception of red blood cells, all mammalian cells, including blastomeres, are capable of undergoing PCD and constitutively express all the proteins required to run the death program.

## Materials and Methods

### Animals and Materials

Sprague-Dawley rats and (Balb/c × C57Bl/6) F1 mice were purchased from Harlan UK Ltd. (Bicester, UK) and maintained in the University College London Animal Facility. Outbred PO (Pathology, Oxford) mice were obtained from the Animal Facility in the ICRF Developmental Biology Unit. STS was a gift from Dr. H. Nakano, Tokyo Research Laboratories

(Kyowa Hakko Kogyo Co. Ltd.). Ethidium homodimer and calcein-AM were purchased from Molecular Probes, Inc. (Eugene, OR). Cell culture media, antibiotics, FCS, terminal deoxynucleotidyl transferase (TdT), and TdT buffer were from GIBCO-BRL (Gaithersburg, MD). Biotin-16-dUTP was from Boehringer Mannheim GmbH (Germany), and streptavidin-fluorescein was from Amersham (UK). All other reagents were from Sigma Chem. Co. (St. Louis, MO), unless indicated.

### Organ Explant Cultures

Neonatal rats were killed by decapitation and various organs were removed and placed in cold L-15 medium; the organs were cut into small pieces (~500  $\mu\text{m}$  on each side) with a hemostat-held blade. Adult and newborn (Balb/c × C57Bl/6) F1 mice were killed and their organs removed as above; small cylindrical pieces of the organs were obtained using a 2-mm punch biopsy needle, except for newborn heart and kidney, which were cut in half with a sharp blade. Rat tissues were cultured at 37°C in a 10% CO<sub>2</sub> incubator on polycarbonate filters (Nucleopore, Pleasanton, CA, 0.8  $\mu\text{m}$  pore size) floating in the wells of 24-well Falcon culture plates in 700  $\mu\text{l}$  of Dulbecco's Modified Eagle's Medium containing 10% FCS and gentamycin (DMEM/FCS). After 48 h, STS (1  $\mu\text{M}$ ) and CHX (10  $\mu\text{g/ml}$ ) were added to the appropriate wells and the cultures continued for an additional 18 h. Mouse tissues were cultured on 1% agarose disks (5 mm in diameter) in 35-mm Falcon bacteriological dishes. The agarose disks (two per dish) were preincubated in 500  $\mu\text{l}$  of DMEM/FCS for 30 min at 37°C in 10% CO<sub>2</sub> before the tissue was added. After 30–60 min, CHX was added to the appropriate dishes to a final concentration of 10  $\mu\text{g/ml}$ , and 30 min later, STS was added to the same dishes to a final concentration of 1 or 10  $\mu\text{M}$ , and the cultures were continued for 1 or 2 d. We showed previously that this concentration of CHX inhibited the incorporation of <sup>35</sup>S-labeled methionine and cysteine into proteins in GM701 cells by more than 95% (Jacobson et al., 1994).

### Frozen Sections and Propidium Iodide and TUNEL Labeling of Explants

Both rat and mouse explants were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 18–48 h and then cryoprotected in 1 M sucrose in the same buffer for 1 d. The tissues were then imbedded in OCT compound (Miles, Inc., West Haven, CT), frozen in liquid nitrogen, and 6–10  $\mu\text{m}$  sections were cut with a Bright cryostat and collected onto APES- or gelatin-coated glass slides. The sections were labeled with propidium iodide (PI) to visualize all of the nuclei, as previously described (Barres et al., 1992). In some cases they were also labeled by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) technique, as described by Gavrieli et al. (1992), with some modifications, using streptavidin-fluorescein as previously described (Ishizaki et al., 1994). The sections stained with PI alone were examined in a Zeiss Universal fluorescence microscope, and the proportion of pyknotic nuclei was counted in representative fields. The doubly stained sections were examined in an MRC-1000 laser-scanning, fluorescence, confocal microscope (Bio-Rad Labs, Hercules, CA). About 6–12 consecutive 1- $\mu\text{m}$  optical sections were summed, and both the TUNEL and PI images of representative fields were printed on a Sony VP860CE printer. The proportion of apoptotic cells was determined by counting the TUNEL-positive cells on the micrographs and dividing by the PI-labeled nuclei in the corresponding micrographs.

### Eggs and Blastomeres

For experiments on outbred PO blastomeres, oestrous females were selected by external inspection (Champlin et al., 1973), paired overnight with males of proven fertility, and checked for the presence of a vaginal plug the next morning. Stages between the 2–4-cell and morula were flushed from the isolated reproductive tract on the second or third day after mating (Hogan et al., 1986). The zona pellucida was dissolved with Tyrode's saline that had been acidified to pH 2.5 with HCl (Nichols and Gardner, 1984). Morulae were decompacted by incubation in Ca<sup>2+</sup>-free OC medium containing 0.2% (wt/vol) ethylene glycol bis-( $\beta$ -amino ethyl ether) N,N,N',N'-tetraacetic acid (Gardner and Nichols, 1991) for 10 min at 37°C before being dissociated by gentle pipetting in a siliconized micropipette. The isolated blastomeres were cultured in 300- $\mu\text{l}$  drops of MTF medium (Lane and Gardner, 1992) at 37°C for 18 h; in some cases STS was present at 1  $\mu\text{M}$ , in the presence or absence of CHX (10  $\mu\text{g/ml}$ ), which was added 30 min before the STS.

In experiments on (Balb/c × C57Bl/6) F1 mouse blastomeres, 6–8-wk-old

female mice were kept in the dark from 8 pm to 8 am and in light from 8 am to 8 pm. At noon they were injected intraperitoneally with five international units (IU) of pregnant mare's serum gonadotropin (PMSG; G-4877, Sigma). 48 h later, five IU of human chorionic gonadotropin (hCG; CG-5, Sigma) was injected. For mating, 1–2 injected female mice were caged overnight with one adult male of the same strain. Females that had mated were used for the recovery of zygotes or 2–4-cell embryos, and unmated females were used for the recovery of unfertilized eggs (Hogan et al., 1986). Groups of eggs or 1–4-cell embryos, with the zona intact, were cultured in 300- $\mu$ l drops of M6 medium (Hogan et al., 1986) at 37°C for 22–60 h in 5% CO<sub>2</sub>, in the presence or absence of STS (10  $\mu$ M) or STS plus CHX (10  $\mu$ g/ml).

Cell viability was assessed by staining with the vital dye bisbenzimidazole (HOECHST 33342) (4  $\mu$ g/ml), which labels the nucleus, calcein-AM (2.5  $\mu$ M), which labels the cytoplasm of live cells, and ethidium homodimer (4  $\mu$ M), which labels the nucleus in cells in which plasma membrane integrity has been lost, as previously described (Jacobson et al., 1994). The cells were examined in an Olympus OMT2 inverted fluorescence microscope at a final magnification of 400.

In the cases where TUNEL labeling was used, the embryos were transferred to PBS containing 1 mg/ml polyvinyl pyrrolidone (PBS/PVP) on siliconized watch glasses. An equal volume of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), containing 1 mg/ml PVP was added, and the embryos were incubated for 10 min at room temperature followed by two washes in PBS/PVP. The embryos were then incubated for 15 min at room temperature in 0.5% pronase (nuclease-free, Calbiochem, San Diego, CA) in PBS/PVP to remove the zona and washed twice in PBS/PVP, at which point most of the embryos broke apart into single blastomeres. The blastomeres were transferred to poly-L-lysine (PLL)-coated 13-mm glass coverslips and fixed with 4% paraformaldehyde in 0.1 M PB for 30 min at room temperature. They were then washed once in distilled water, allowed to dry, and then stored dry at –20°C until TUNEL staining, as described above.

### Blastocysts and Inner Cell Mass

Blastocysts were recovered from timed pregnant outbred mice and the zona pellucida was removed with acidified Tyrode's saline. ICMs were obtained from blastocysts by immunosurgery (Solter and Knowles, 1975; Nichols and Gardner, 1984). In brief, after removal of the zona, the blastocysts were allowed to recover for 1 h at 37°C in  $\alpha$ -medium containing 10% FCS. They were rinsed in PBS and then PB plus FCS, and were then incubated in heat-inactivated rabbit anti-mouse antiserum (diluted 1:9 in PB) for 45 min at 37°C. They were rinsed three times for 1 min each in PB plus FCS and then incubated in rat complement (diluted 1:9 in PB) for 8 min at 37°C. The blastocysts were then transferred to  $\alpha$ -medium containing 10% FCS for at least 45 min at 37°C to recover, before being gently pipetted to remove the lysed trophectoderm from the intact ICM. The zonaless blastocysts or isolated inner cell masses were cultured in  $\alpha$ -medium in the presence or absence of 1  $\mu$ M STS, or 1  $\mu$ M STS plus 10  $\mu$ g/ml CHX, at 37°C in 5% CO<sub>2</sub> in Sterilin bacteriological plastic dishes for 18 h. Cell viability was assessed as for blastomeres.

### Human Red Blood Cells

About 20  $\mu$ l of human blood was obtained by finger prick and washed once in PBS containing 0.5 mM EDTA and once in DMEM. The red blood cells were counted in a haemocytometer and 2  $\times$  10<sup>6</sup> cells were cultured in 2 ml DMEM/FCS, either alone or with 10  $\mu$ M STS or 10  $\mu$ M STS plus 10  $\mu$ g/ml CHX, in 35-mm Falcon tissue culture dishes at 37°C in 10% CO<sub>2</sub> for up to 2 wk. The cells were examined with phase contrast optics in an inverted Nikon TMA microscope.

## Results

### Neonatal Organs

In initial experiments, small pieces of neonatal rat kidney, liver, lung, heart, muscle, small intestine, or skin were cultured on floating polycarbonate filters for 48 h in DMEM/FCS, before STS was added to a final concentration of 1  $\mu$ M. After an additional 24 h, the explants were fixed in formaldehyde, cut into frozen sections, and stained with propidium iodide (PI) to visualize the nuclei. In all cases, most of

the cells in the STS-treated explants had a pyknotic, and often fragmented nucleus, whereas fewer than 20% of the cells in untreated explants were pyknotic (not shown).

We then tested similar explants of neonatal mouse heart, kidney, lung, muscle, pancreas, liver or skin, either on floating polycarbonate filters or on agarose platforms. In these experiments the explants were treated with 1  $\mu$ M or 10  $\mu$ M STS in the presence or absence of 10  $\mu$ g/ml CHX, for either 1 or 2 d. The results in all organs were similar. In control cultures maintained in the absence of STS and CHX, there were always some cells with a pyknotic nucleus, but most of the cells appeared normal. By contrast, in cultures treated with 1 or 10  $\mu$ M STS alone, or with STS and CHX, most of the cells had a pyknotic, and sometimes fragmented, nucleus. To quantify the PCD induced by STS plus CHX, explants were double-labeled by the TUNEL technique to detect DNA fragmentation and with PI to visualize all the nuclei. The proportions of PI-labeled nuclei that were TUNEL-positive were counted in confocal fluorescence micrographs. In all cases the majority of the nuclei in treated explants were TUNEL-positive; only a minority of the nuclei in untreated control explants were TUNEL-positive. In explants treated with 10  $\mu$ M STS and 10  $\mu$ g/ml CHX for 2 d, almost all of the nuclei were TUNEL-positive, as shown for representative experiments in Table I and Fig. 1. These findings suggest that the great majority of the nucleated cells in developing organs are capable of undergoing PCD and apparently do not have to make new proteins to do so.

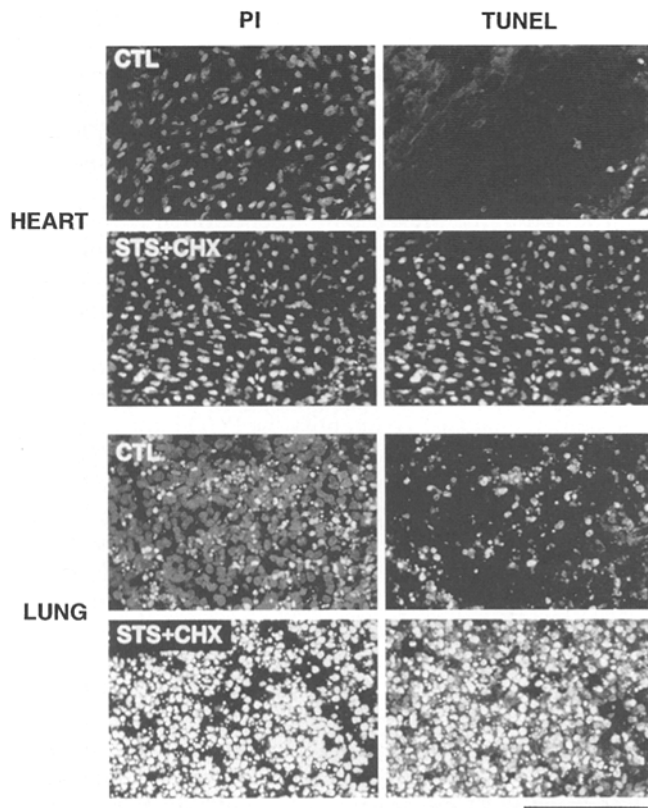
### Adult Organs

To determine whether cells in adult tissues respond similarly to those in developing tissues, we cultured explants of adult mouse kidney, lung and ear pinna on agarose platforms and treated them as described above for developing tissues. Although the cells in the adult tissues were gener-

Table I. (STS + CHX)-induced PCD in Explants of Newborn Mouse Organs

Organ	Treatment duration	Staurosporine	PCD
	<i>d</i>	$\mu$ M	%
Heart	1	0	37
	"	1	85
	"	10	99
Skin	1	0	26
	"	1	69
	"	10	78
	2	0	41
	"	1	65
Lung	"	10	100
	1	0	22
	"	1	55
	"	10	64
	2	0	31
	"	1	96
	"	10	98

Pieces of newborn (Balb/c  $\times$  C57Bl/6) F1 mouse organs were cultured on agarose platforms in DMEM/FCS. In STS-treated explants, CHX was added (to a final concentration of 10  $\mu$ g/ml) 30 min before adding the STS. Frozen sections of the explants were double-labeled with PI and by the TUNEL technique, and the proportions of PI-labeled nuclei that were TUNEL-positive were counted in confocal fluorescence micrographs. Between 300 and 3,000 cells in multiple representative fields were assessed for each organ and condition.



**Figure 1.** Confocal fluorescence micrographs of (STS + CHX)-induced PCD in explant cultures of newborn mouse heart (*upper four panels*) and lung (*lower four panels*). The cultures were treated and processed as in Table I. The heart tissue was cultured for 1 d in the presence or absence of 10  $\mu$ M STS and 10  $\mu$ g/ml CHX, while the lung tissue was cultured for 2 d in the presence or absence of 1  $\mu$ M STS and 10  $\mu$ g/ml CHX. Bar, 100  $\mu$ m.

ally less sensitive to STS plus CHX than cells in developing tissues, when treated with 10  $\mu$ M STS in the presence of CHX for 2 d, most of the cells had a pyknotic and sometimes fragmented nucleus, which was labeled when analyzed by the TUNEL technique (Table II). The amount of PCD in both control and treated specimens varied across the explant. In control explants, however, there were many regions where most of the cells were alive and only rare regions where most of the cells were dead; in treated cultures, by contrast, there were no regions where most of the cells were alive and many regions where almost all of the cells were dead (Fig. 2). The only cells that seemed resistant to treatment with STS and CHX were chondrocytes in the pinna, but this could be because the drugs failed to penetrate the cartilage matrix (see Discussion). Thus, even in adult tissues, the majority of nucleated cells seem to be capable of undergoing PCD and apparently do not have to make new proteins to do so.

### Blastomeres and Eggs

As mouse blastomeres do not require signals from other cells to survive *in vitro*, it was of interest to test whether they could be induced to undergo PCD when treated with STS in the presence or absence of CHX. Blastomeres dissociated from 2- or 4-cell mouse embryos did not die when cul-

**Table II.** (STS + CHX)-induced PCD in Explants of Adult Mouse Organs

Organ	Treatment duration	Staurosporine	PCD
	<i>d</i>	$\mu$ M	%
Kidney	1	0	3
	"	10	80
	2	0	31
Ear pinna	"	10	88
	1	0	10
	"	10	46
Lung	2	0	27
	"	10	70
	2	0	35
	"	10	100

Pieces of adult (Balb/c  $\times$  C57Bl/6) F1 mouse organs were cultured and assayed as in Table I. Between 100 and 1,400 cells in multiple representative fields were assessed for each organ and condition.

tured for 18 or 24 h with 1  $\mu$ M STS, with or without CHX: the cells remained phase-bright, excluded ethidium homodimer (indicating plasma membrane integrity), had a normal looking nucleus when stained with bisbenzimidazole, and were not labeled when analyzed by the TUNEL technique (not shown). Blastomeres in intact 4-cell embryos behaved similarly, even with 10  $\mu$ M STS (Table III), as did freshly ovulated mouse eggs (not shown). Thus, blastomeres and oocytes are unusually resistant to the lethal effects of STS and CHX, as all other normal, developing nucleated cell types we have tested undergo PCD when treated with these doses for this time in dissociated-cell culture.

When eggs or 1–4-cell embryos were treated with 10  $\mu$ M STS, with or without CHX, for 26–60 h, however, most of the cells died (Table III), as judged by their abnormal morphology when viewed by phase contrast microscopy, their failure to fluoresce when exposed to calcein, their staining with ethidium homodimer, their pyknotic nucleus visualized by bisbenzimidazole or PI staining, and by their staining when labeled by the TUNEL technique (Fig. 3). Thus, blastomeres and eggs seem to be capable of undergoing PCD and do not need to make new proteins to do so.

### Blastocysts

By the 32-cell stage, mouse blastomeres have developed into two distinct cell types, ICM cells, which will give rise to the cells of the embryo proper, and trophoblast cells, which will give rise to cells of the extraembryonic membranes. When 32–64-cell blastocysts were treated with 1  $\mu$ M STS and 10  $\mu$ g/ml CHX for 18 h, more than 90% of the cells died with the characteristic morphological features of apoptosis (Fig. 4) indicating that both ICM and trophoblast cells are more sensitive to these agents than are blastomeres. When isolated ICMs were treated in this way, all of the cells died with the features of apoptosis (Fig. 4). Thus, blastomeres become more sensitive to (STS plus CHX)-induced PCD after they differentiate into ICM and trophoblast.

### Human Red Blood Cells

When human red blood cells were cultured in DMEM/FCS at  $10^6$  cells/ml, they survived as normal-appearing,

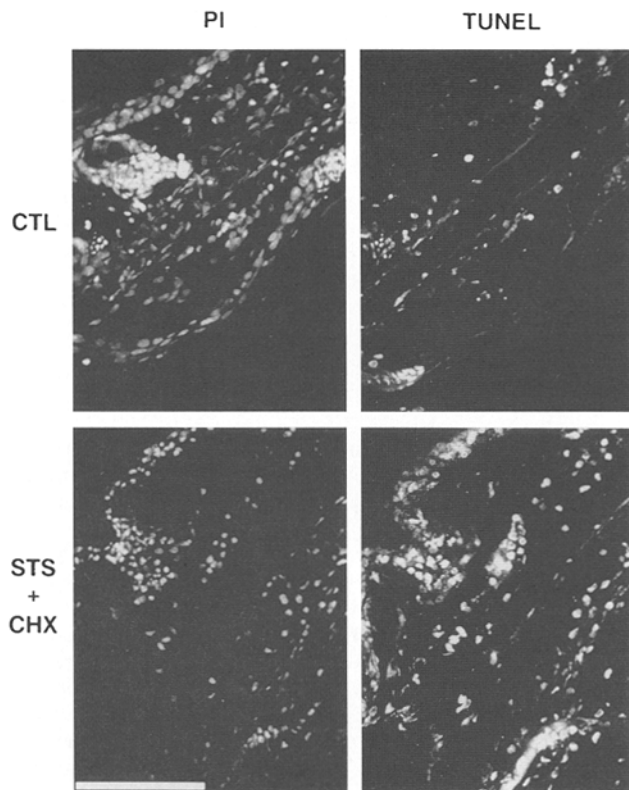


Figure 2. Confocal fluorescence micrographs of (STS + CHX)-induced PCD in explant cultures of adult mouse pinna. The tissues were cultured for 2 d and processed as in Table I. STS was used at 10  $\mu$ M and CHX at 10  $\mu$ g/ml. Bar, 100  $\mu$ m.

phase-bright, biconcave cells for at least a week. After this time, the cells began to die, and all were dead by 2 wk. Dead cells were easily distinguished as small, phase dark, round ghosts. Treatment with 10  $\mu$ M STS, with or without CHX, had no effect on the morphology or survival of the cells (not shown).

## Discussion

We find that the vast majority of cells in explant cultures of 8/8 types of newborn organs and 3/3 types of adult organs die with the characteristic features of apoptosis when treated with high concentrations of STS in the presence of CHX. The only cell type that seems to resist this treatment is the chondrocyte in explants of adult pinna. It is likely, however, that this apparent resistance of the chondrocytes reflects the failure of the STS and/or CHX to penetrate the extracellular matrix of the cartilage, as we showed previously that chondrocytes dissociated from adult rat sternum undergo apoptosis when treated *in vitro* with 1  $\mu$ M STS for 1 d (Ishizaki et al., 1994). The requirement for a higher concentration of STS and a longer treatment time for cells to undergo PCD in explants of adult organs compared to neonatal organs may also reflect differences in drug penetration. Taken together with previous results obtained with various cell lines and dissociated primary cells (see references in Introduction), our present findings suggest that all nucleated mammalian cells, both in developing and mature

Table III. STS and (STS + CHX)-induced PCD in Mouse Embryos and Eggs

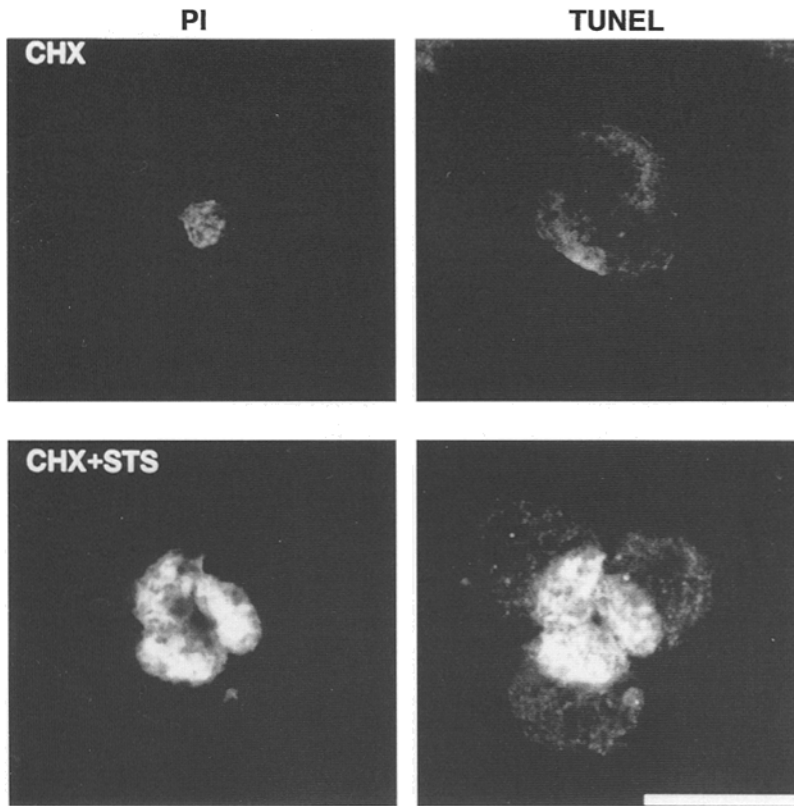
Stage	Treatment duration	Treatment	Dead/Total	Cell death %
4-Cell	22	0	0/99	0
	"	CHX	0/53	0
	"	STS	3/52	6
	"	CHX + STS	0/48	0
1-Cell	36	0	0/84	0
	"	CHX	0/22	0
	"	STS	21/21	100
	"	CHX + STS	23/26	89
2-Cell	37	0	3/46	2
	"	CHX	3/49	6
	"	STS	57/58	98
	"	CHX + STS	47/49	96
4-Cell	36	0	11/204	5
	"	CHX	0/56	0
	"	STS	49/53	93
	"	CHX + STS	66/69	96
Egg	28	0	2/42	5
	"	STS	34/45	76
	36	CHX	0/24	0
"	CHX + STS	22/23	96	

Embryos and eggs were flushed from the reproductive tract of mated or unmated superovulated female (Balb/c  $\times$  C57Bl/6) F1 mice. They were cultured with the zona pellucida intact in 300  $\mu$ l drops of M6 medium. STS was used at 10  $\mu$ M and CHX at 10–30  $\mu$ g/ml. Cell death was indicated by an abnormal morphology when viewed by phase-contrast optics, fluorescent staining with ethidium homodimer, lack of calcein fluorescence, and nuclear pyknosis visualized by bisbenzimidazole staining.

organs, are capable of undergoing PCD and constitutively express all of the protein components required to execute the death program. The fact that STS is not a physiological inducer of PCD and that we do not know how it activates PCD does not affect this conclusion. In those cases where inhibitors of RNA or protein synthesis inhibit PCD, it is presumably because macromolecular synthesis is required to activate the death program rather than to execute it.

Even blastomeres and ovulated eggs, both of which are relatively resistant to STS treatment, die when the concentration of STS is increased from 1  $\mu$ M to 10  $\mu$ M and treatment time is extended from 18–22 h to 36–60 h, either in the presence or absence of CHX. In the case of blastomeres, at least, in the presence or absence of CHX, the cells die with the characteristic features of apoptosis, suggesting that these cells too are capable of undergoing PCD and constitutively express the proteins required to execute the death program. It seems, therefore, that the reason blastomeres do not require extracellular signals to survive (Biggers et al., 1971) is not because they lack a functional death program, but rather because they can keep the death program suppressed in the absence of such signals. It is likely that eggs are similar to blastomeres in these respects. Because they are arrested in metaphase of the second meiotic division, however, eggs do not have a discrete nucleus, so it is difficult to assess whether the death induced by STS is PCD, although this seems likely.

When blastomeres develop into ICM and trophecto-



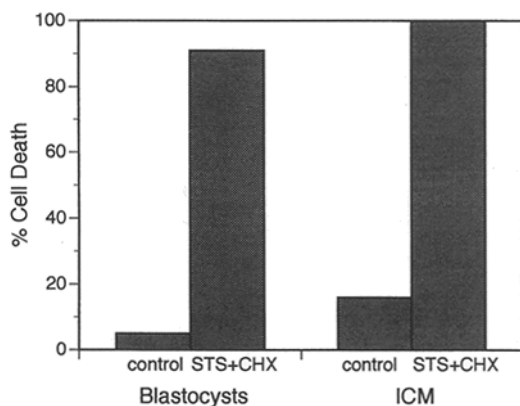
**Figure 3.** Confocal fluorescence micrographs of (STS + CHX)-induced PCD in blastomeres. Mouse embryos (4–6 cells) were cultured for 36 h in medium alone or STS alone (not shown), or in CHX (10  $\mu\text{g/ml}$ ) or CHX plus STS (10  $\mu\text{M}$ ). The zona pellucida was removed and the blastomeres double-labeled with PI and the TUNEL technique as described in Materials and Methods. A TUNEL-negative blastomere that was treated with CHX alone is shown in the upper panels. Three TUNEL-positive blastomeres that were treated with CHX and STS are shown in the lower panels. Blastomeres treated with medium alone were also TUNEL-negative. Bar, 25  $\mu\text{m}$ .

derm, we find that they become just as sensitive to (STS plus CHX)-induced PCD as other developing cells. The molecular basis for this change in susceptibility remains to be determined. The change in susceptibility is consistent with the findings that substantial numbers of ICM, and possibly trophoblast cells, undergo PCD in normal development (El-Shershaby and Hincliffe, 1974), whereas blastomeres apparently do not.

Perhaps not surprisingly, mature human erythrocytes do not die when treated with 10  $\mu\text{M}$  STS in the presence or

absence of CHX. Thus far, they are the only mammalian cells shown not to do so. These cells lack all organelles and presumably do not have the molecular machinery required for PCD. It is not simply the lack of a nucleus that is responsible for their resistance, as anucleate cytoplasts are able to undergo PCD (Jacobson et al., 1994; Schulze-Osthoff et al., 1994; Nakajima et al., 1995).

Much of what is known about the molecular mechanism of PCD came originally from genetic studies of the nematode *C. elegans*, which identified two genes, *ced-3* and *ced-4*, that are required for all the PCDs that occur during development: if either gene is inactivated by mutation, none of these cell deaths occur (Ellis and Horvitz, 1986). Whereas *ced-4* encodes a novel protein (Yuan and Horvitz, 1992), *ced-3* encodes a member of the IL-1 $\beta$  converting enzyme (ICE) family of cysteine proteases (Yuan et al., 1993). One or more members of this family also seem to play important roles in PCD in vertebrate cells: *ced-3* and other family members can induce PCD when ectopically expressed in mammalian cells (Gagliardini et al., 1994; Miura et al., 1993; Wang et al., 1994; Fernandes-Alnemri et al., 1994; Kumar et al., 1994), and inhibitors of these proteases can inhibit PCD in vertebrate cells (for review see Martin and Green, 1995), including STS-induced PCD (Cain et al., 1995; Mashima et al., 1995; Zhu et al., 1995, and accompanying paper). Another *C. elegans* gene, *ced-9*, acts as a brake on the death program: when it is inactivated by mutation, most of the cells in the developing worm undergo *ced-3*- and *ced-4*-dependent PCD (Hengartner et al., 1992). The latter finding suggests that *ced-3* and *ced-4*, and the other genes required to execute the death program, are constitutively expressed in most, and perhaps all, cells in the developing worm. It may be, therefore, that consti-



**Figure 4.** (STS + CHX)-induced PCD in blastocysts and ICMs. Blastocysts at the 32–64-cell stage were flushed from the reproductive tract of timed pregnant PO mice, and the zona pellucida was removed with acidified Tyrode's saline. ICMs were isolated by immunosurgery. The blastocysts and ICMs were cultured in  $\alpha$ -medium in the presence or absence of 1  $\mu\text{M}$  STS and 10  $\mu\text{g/ml}$  CHX for 18 h, and cell viability was assessed as in Table III.



tutive expression of the machinery for PCD including the relevant members of Ced-3/ICE family of proteases, is a general feature of animal cells.

Two novel cell death genes, *reaper* (White et al., 1994) and *hid* (Grether et al., 1995), have been identified in *Drosophila*. A DNA deletion that eliminates both genes eliminates all of the PCDs that occur during normal fly development, yet PCD can occur if the mutant flies are exposed to a very high dose of x-rays (White et al., 1994). Thus, these death genes apparently encode proteins that activate the death program rather than execute it. Moreover, *reaper* is transcriptionally activated 1–2 h before PCD, suggesting that the activation of the death machinery in flies normally depends on RNA and protein synthesis. This seems likely to be the case also for many of the normal cell deaths that occur in developing vertebrates as well (Tata, 1966; Oppenheim et al., 1990; Coucouvanis and Martin, 1995), although a homologue of *reaper* has yet to be identified in vertebrates.

We thank Dr. H. Nakano for the generous supply of STS.

M.D. Jacobson is supported by a British Telecom-sponsored grant from the Multiple Sclerosis Society (UK). T.J. Davies is supported by the Imperial Cancer Research Fund. R.L. Gardner is supported by the Imperial Cancer Research Fund and Royal Society.

Received for publication 30 November 1995 and in revised form 2 January 1996.

## References

- Barres, B.A., I.K. Hart, H.S.R. Coles, J.F. Burne, J.T. Voyvodic, W.D. Richardson, and M.C. Raff. 1992. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70:31–46.
- Baserga, R. 1985. *The Biology of Cell Reproduction*. Harvard University Press, Cambridge, MA. 256 pp.
- Bertrand, R., E. Solary, P. O'Connor, K.W. Kohn, and Y. Pommier. 1994. Induction of a common pathway of apoptosis by staurosporine. *Exp. Cell Res.* 211:314–321.
- Biggers, J.D., W.K. Whitten, and D.G. Whittingham. 1971. The culture of mouse embryos in vitro. In *Methods in Mammalian Embryology*. J.C. Daniel, editor. Freeman, San Francisco, CA. 86–116.
- Cain, K., S.H. Inayat-Hussein, C. Couet, and G.M. Cohen. 1996. A cleavage site-directed inhibitor of interleukin-1 $\beta$ -converting enzyme-like proteases inhibits apoptosis in primary cultures of rat hepatocytes. *Biochem. J.* 314:27–32.
- Champlin, A.K., D.L. Dorr, and A.H. Gates. 1973. Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. *Biol. Reprod.* 8: 491–494.
- Coucouvanis, E., and G.R. Martin. 1995. Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. *Cell* 83:279–287.
- Duke, R.C., and J.J. Cohen. 1986. IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. *Lymphokine Res.* 5:289–299.
- El-Shershaby, A.M., and J.R. Hinchliffe. 1974. Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscopic study of dead cells and their fate. *J. Embryol. Exp. Morphol.* 31:643–654.
- Ellis, H.M., and H.R. Horvitz. 1986. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 44:817–829.
- Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri. 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 $\beta$ -converting enzyme. *J. Biol. Chem.* 269:30761–30764.
- Gagliardini, V., P.A. Fernandez, R.K. Lee, H.C. Drexler, R.J. Rotello, M.C. Fishman, and J. Yuan. 1994. Prevention of vertebrate neuronal death by the *crmA* gene. *Science (Wash. DC)* 263:826–828.
- Gardner, R.L., and J. Nichols. 1991. An investigation of the fate of cells transplanted orthotopically between morulae/nascent blastocysts in the mouse. *Hum. Reprod.* 6:25–35.
- Gavrieli, Y., Y. Sherman, and S.A. Ben Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119:493–501.
- Glucksman, A. 1951. Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* 26: 59–86.
- Grether, M.E., J.M. Abrams, J. Agapite, K. White, and H. Steller. 1995. The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev.* 9:1694–1708.
- Harrington, E., A. Fanidi, M. Bennett, and G. Evan. 1994. Modulation of Myc-induced apoptosis by specific cytokines. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3286–3295.
- Hengartner, M.O., R.E. Ellis, and H.R. Horvitz. 1992. *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. *Nature (Lond.)* 356: 494–499.
- Hogan, B., F. Constantini, and E. Lacey. 1986. *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Ishizaki, Y., J.F. Burne, and M.C. Raff. 1994. Autocrine signals enable chondrocytes to survive in culture. *J. Cell Biol.* 126:1069–1077.
- Ishizaki, Y., J.F. Burne, and M.C. Raff. 1995. Programmed cell death by default in embryonic cells, fibroblasts and cancer cells. *Mol. Biol. Cell.* 6:1443–1458.
- Ishizaki, Y., J.T. Voyvodic, J.F. Burne, and M.C. Raff. 1993. Control of lens epithelial cell survival. *J. Cell Biol.* 121:899–908.
- Jacobson, M.D., J.F. Burne, M.P. King, T. Miyashita, J.C. Reed, and M.C. Raff. 1993. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature (Lond.)* 361:365–369.
- Jacobson, M.D., J.F. Burne, and M.C. Raff. 1994. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1899–1910.
- Kumar, S., M. Kinoshita, M. Noda, N.G. Copeland, and N.A. Jenkins. 1994. Induction of apoptosis by the mouse *Nedd2* gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1 $\beta$ -converting enzyme. *Genes Dev.* 8:1613–1626.
- Lane, M., and D.K. Gardner. 1992. Effect of incubation volume and embryo density on the development of mouse embryos in vitro. *Hum. Reprod.* 7:558–562.
- Martin, D.P., R.E. Schmidt, P.S. DiStefano, O.H. Lowry, J.G. Carter, and E.M. Johnson. 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* 106: 829–844.
- Martin, S., and D. Green. 1995. Protease activation during apoptosis: death by a thousand cuts? *Cell* 82:349–352.
- Martin, S.J. 1993. Apoptosis: suicide, execution or murder? *Trends Cell Biol.* 3: 141–144.
- Mashima, T., M. Naito, S. Kataoka, H. Kawai, and T. Tsuruo. 1995. Aspartate-based inhibitor of interleukin-1 $\beta$ -converting enzyme prevents antitumor agent-induced apoptosis in human myeloid leukemia U037 cells. *Biochem. Biophys. Res. Commun.* 209:907–915.
- Miura, M., H. Zhu, R. Rotello, E.A. Hartwig, and J. Yuan. 1993. Induction of apoptosis in fibroblasts by IL-1 $\beta$ -converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* 75:653–660.
- Nakajima, H., P. Golstein, and P.A. Henkart. 1995. The target cell nucleus is not required for cell-mediated granzyme- or Fas-based cytotoxicity. *J. Exp. Med.* 181:1905–1909.
- Nichols, J., and R.L. Gardner. 1984. Heterogeneous differentiation of external cells in individual isolated early mouse inner cell masses in culture. *J. Embryol. Exp. Morph.* 80:225–240.
- Oppenheim, R.W., D. Prevette, M. Tytell, and S. Homma. 1990. Naturally occurring and induced neuronal death in the chick embryo in vivo requires protein and RNA synthesis: evidence for the role of cell death genes. *Dev. Biol.* 138:104–113.
- Raff, M.C. 1992. Social controls on cell survival and cell death. *Nature (Lond.)* 356:397–400.
- Raff, M.C., B.A. Barres, J.F. Burne, H.S. Coles, Y. Ishizaki, and M.D. Jacobson. 1993. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science (Wash. DC)* 262:695–700.
- Reed, J.C. 1994. Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* 124:1–6.
- Schulze-Osthoff, K., H. Walczak, W. Dröge, and P.H. Kramer. 1994. Cell nucleus and DNA fragmentation are not required for apoptosis. *J. Cell Biol.* 127:15–20.
- Solter, D., and B.B. Knowles. 1975. Immunotherapy of mouse blastocyst. *Proc. Natl. Acad. Sci. USA.* 72:5099–5102.
- Tamaoki, T., and H. Nakano. 1990. Potent and specific inhibitors of protein kinase C of microbial origin. *Bio/Technology.* 8:732–735.
- Tata, J.R. 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. *Dev. Biol.* 13:77–94.
- Thornberry, N., D. Miller, and D. Nicholson. 1995. Interleukin-1 $\beta$ -converting enzyme and related proteases as potential targets in inflammation and apoptosis. *Perspectives in Drug Discovery and Design.* 2:389–399.
- Wang, L., M. Miura, L. Bergeron, H. Zhu, and J. Yuan. 1994. Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78:739–750.
- White, K., M.E. Grether, J.M. Abrams, L. Young, K. Farrell, and H. Steller. 1994. Genetic control of programmed cell death in *Drosophila*. *Science (Wash. DC)* 264:677–683.
- Yuan, J., and H.R. Horvitz. 1992. The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development.* 116:309–320.
- Yuan, J., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 $\beta$ -converting enzyme. *Cell* 75:641–652.
- Zhu, H., H.O. Fearnhead, and G.M. Cohen. 1995. An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP-1 cells. *FEBS Lett.* 374:303–308.