The implications of a unified theory of programmed cell death, polyamines, oxyradicals and histogenesis in the embryo

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ABSTRACT Programmed cell death (apoptosis) is the ubiquitous biological phenomenon of intentional cell death that eliminates redundant cells, changes phenotypic composition during histogenesis, provides form during morphogenesis and balances mitosis in renewing tissues. This form of cell death is controlled by a genetic program(s) that kills the targeted cell without causing subsequent inflammation. Malignant cells implanted into the appropriate regulatory field in the embryo will lose their malignant phenotype yet retain the capacity for proliferation and differentiation. This embryonic regulation of cancer requires simultaneous contact with specific structures on the surfaces of normal cells and exposure to soluble, extracellular signals. During studies to identify such soluble factors in the blastocyst, extracellular hydrogen peroxide was discovered in the blastocele fluid. Current evidence indicates that this hydrogen peroxide causes apoptosis of inner cell mass cells destined to develop into trophectoderm — the first apoptotic event during mammalian development which likely prevents the formation of ectopic trophectoderm in the soon-to-appear germ layers (histogenesis). The evidence also suggests that the hydrogen peroxide is generated during the oxidation of extracellular polyamines by a family of enzymes called amine oxidases. The components of this mechanism are also present in the mammalian epidermis, where they are proposed to control the survival of basal cell progeny and hence epidermal homeostasis (essentially controlling the production of tissue mass). This mechanism causes not only apoptosis *in vivo*, but also the unwanted and artefactual cell death *in vitro* known as the crisis of spontaneous transformation. These data suggest a novel link between polyamines and apoptosis, a link that has practical as well as theoretical implications. The data also explain why negative regulators of tissue renewal, the so-called «chalones», have been so difficult to purify: polyamines are the stable, non-toxic storehouses of regulatory peroxide which have no activity *per se*, while the highly reactive and short-lived peroxide is the regulatory agent which would not be identified by classical biochemical approaches. Furthermore, negative regulators of tissue renewal were expected to be reversibly-acting, non-toxic compounds, so the finding that a cytotoxic compound regulates renewing populations by killing some but not all of the target cells was unexpected indeed. However, since these discoveries, regulatory functions mediated by paracrine/autocrine factors have been ascribed to other reactive or volatile compounds, such as nitrous oxide radical and carbon dioxide.

KEY WORDS: *blastocyst, free-radicals, limb bud, apoptosis, amine oxidase, hydrogen peroxide, epidermis*

Introduction

I asked to join Dr. Pierce's laboratory in early 1987, after hearing his lecture on the exciting possibility for a non-cytotoxic cure for malignancies locked within the secrets of embryogenesis (Pierce and Johnson, 1971; Pierce and Speers, 1988). As a pharmacologist trained in an oncology setting, this revelation was a breath of fresh air, which seemed to provide a basis for developing anti-cancer therapeutics with much higher therapeutic indices than was (and is) currently possible. So, I went to Colorado in the hopes of developing embryonic regulation into experimental therapeutics. Little did I, and perhaps even Dr. Pierce with his excellent «nose» for biological systems, foresee what novel cell biology was coming our

Abbreviations used in this paper: ICM, inner cell mass.

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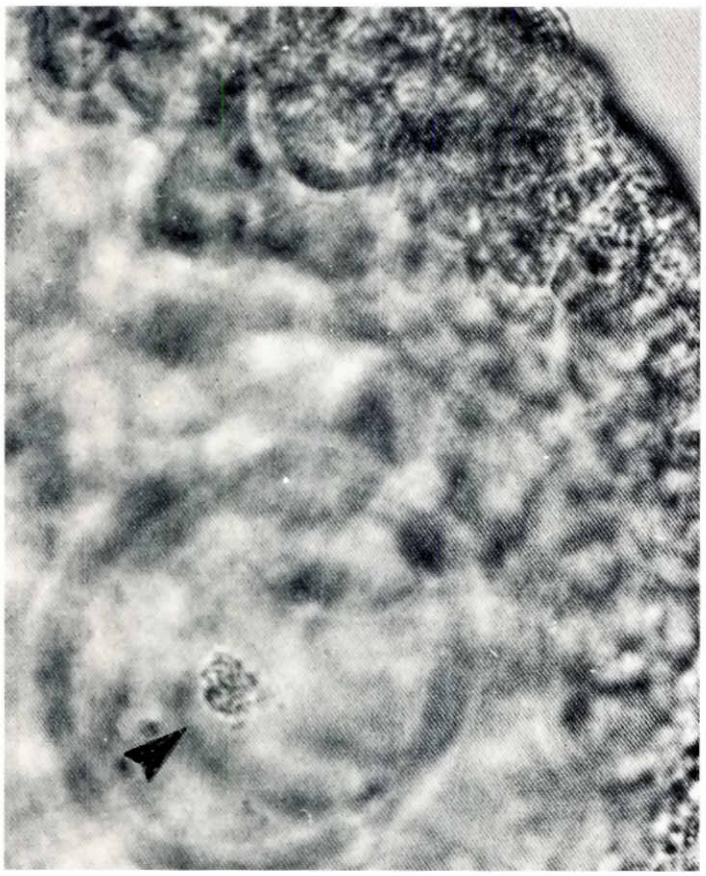

Fig. 1. A single ECa 247 cell (arrowhead) in an evacuated *zona pellucida* carrier has undergone apoptosis as a result of exposure to the blastocoele fluid of this giant blastocyst.

way. Yet the results of these recent studies can answer some classic questions in developmental and cellular biology. Why can't physiological inducers of apoptosis or regulators of tissue renewal be isolated? What mechanism makes possible the death of one cell by apoptosis in the midst of neighboring, viable cells? Why do mouse embryo cells invariably die *in vitro* after 4-6 weeks of serial passaging?

Setting the stage

In 1974, Brinster reported that murine teratocarcinoma cells implanted into blastocysts participated in embryonic development and eventuated chimeric animals (Brinster, 1974). This result suggested that cancer is not immutable, and that the mechanisms were present in the embryo for abrogating the malignant phenotype without affecting proliferative potential and without cytotoxicity. Dr. Pierce's laboratory had developed assays to measure and study such «embryonic regulation» (Pierce *et al.*, 1979, 1984), and, using these assays, had shown that both soluble factors and cell contact between malignant cell and normal cells were required to regulate a cancer cell (Pierce *et al.*, 1984, 1989b). A degree of specificity was found for the intercellular contact: embryonal carcinoma cell lines which lacked potential for becoming trophectoderm in the blastocyst (such as P19) required contact with trophectoderm (Pierce *et al.*, 1989b), yet lines with potential to become trophectoderm in the blastocyst (such as ECa 247) could be regulated by contact with cells of either the inner cell mass or trophectoderm (Pierce *et al.*, 1984, 1989b). Dr. Pierce and I set out to identify the soluble factors that contribute to regulation, since this aspect of regulation was suspected to be simpler than the intercellular contact.

Just couldn't get away from cell death masquerading as inhibition of proliferation: regulation of tissue mass

To assay for the presence of an inhibitor of proliferation in blastocoele fluid, giant blastocysts were employed, into the cavity of which were inserted emptied *zonae pellucidae* carriers containing either ECa 247 or P19 cells, the embryonal carcinoma cell lines that do and do not make trophectoderm after injection into the blastocyst, respectively (Pierce *et al.*, 1989b). Enclosed in the *zonae* carriers, the proliferation rate of the embryonal carcinoma cells could be determined by direct cell counting because they were visible by microscopic observation.

The result of this experiment caused considerable excitement (Table 1). P19 cells proliferated in the blastocoele fluid at control rates. In contrast, the proliferation of ECa 247 cells was slowed 50% in the blastocoele fluid. Thus, blastocoele fluid appeared to contain a lineage-specific inhibitor of proliferation: this activity inhibited only the proliferation of cells destined to enter the trophectodermal layer.

At the same time, we were also trying to develop a cell culture system to screen for soluble factors in the limb bud that would inhibit the proliferation of B16 melanoma cells, which were regulated by day-14 embryonic limbs (Gerschenson *et al.*, 1986). In clonogenic assays, such activity had been found in the low molecular weight fraction of media conditioned by day-14 limb bud cells, eluting near the V_t of a Sephadex G-50 column (Gerschenson *et al.*, 1986). However, unbeknownst at the time, the processing of the assay plates for fixation and staining of the clonogenic colonies was removing an important clue about the mechanism producing apparent inhibition of proliferation — detached, dead cells.

Microscopic examination before removing the culture medium revealed dead B-16 cells within 24 h of adding the limb bud-conditioned medium (Parchment and Pierce, 1989). At highest concentrations, the conditioned medium could kill 1000 B-16 cells

TABLE 1

APPARENT INHIBITION OF PROLIFERATION OF ECa 247 BUT NOT P19 BY BLASTOCOELE FLUID

Cell Line	N_{24}/N_0* (Actual Cell Counts)	
	Experimental	Control
ECa 247	1.6 (57/36)	3.5 (204/58)
P19	2.8 (62/28)	3.0 (103/34)

N_{24} is the number of cells after 24 h and N_0 is the number of cells at the start of the experiment.

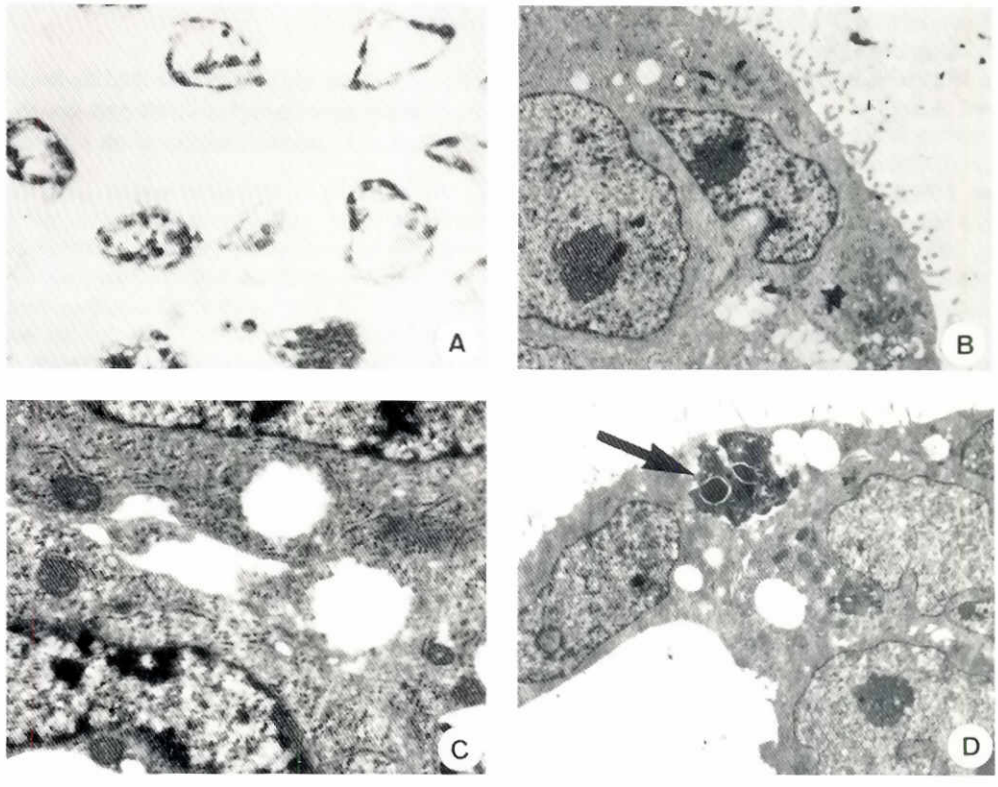

Fig. 2. The ascites form of the embryonal carcinoma C44 used as a source of blastocoele-like fluid because it contains the inducer of apoptosis of ECa 247 cells. (A) *Histologic appearance of embryoid bodies in vivo.* **(B)** *Electron microscopic view near the surface of an embryoid body showing the slender microvilli of the outer layer.* **(C)** *Electron microscopic view of the extensive rough endoplasmic reticulum of a cell lining the cavity of a fluid-filled embryoid body.* **(D)** *A phagocytosed, apoptotic cell (arrow) in the wall of a fluid-filled embryoid body. Apoptotic cells were never seen in the solid aggregates prior to expansion with fluid.*

within 18 h. At intermediate concentrations, the conditioned medium killed some but not all of the target cells; the surviving cells continued to proliferate, and, at the end of the assay, a cell count would therefore indicate inhibition of proliferation. In other words, either the selective death of some but not all of the cells of a population or the inhibition of proliferation within a cell population produces the same net result upon cell number (tissue mass) over time. Only by microscopic observation was it possible to distinguish these two mechanisms (Parchment and Pierce, 1989; Pierce *et al.*, 1989b).

We soon realized that the apparent inhibition of proliferation of ECa 247 in blastocoele fluid might also be due to the same mechanism — the death of some but not all of the target cells. We re-examined this system, but this time only one cell was placed in each *zona* carrier implanted into the blastocoele cavity of a giant blastocyst. The data showed unambiguously that the blastocoele fluid was selectively toxic to ECa 247 but not P19 cells (Fig. 1), killing 42% of the injected ECa 247 cells within 24 h (Pierce *et al.*, 1989b). Thus, we had misinterpreted the initial data; what had been interpreted as inhibition of proliferation was actually due to the death of some but not all of the cells in the target population.

The *in vivo* confirmation with ECa 247 cells of the *in vitro* data with B-16 cells certainly increased our confidence in the relevancy of the *in vitro* B-16 model system, and at that time this confirmation was a deciding factor for choosing to continue this research.

Death of cancer cells in the embryo reflects normal programmed cell death in the embryo

The blastocyst model revealed that the embryo contains a mechanism which kills implanted cancer cells of particular developmental lineages (Pierce *et al.*, 1989b). These data raised the question, why should the embryo have such mechanisms? Surely this mechanism must serve a developmental function in normal embryogenesis and only incidentally kill implanted cancer cells (Parchment and Pierce, 1989). After some intense discussion, it occurred to us that the mechanism responsible for programmed cell death during embryogenesis might be responsible for the death of the cancer cells (Parchment and Pierce, 1989; Pierce *et al.*, 1989b).

Programmed cell death occurs throughout embryogenesis, likely contributing to morphogenesis and histogenesis. It occurs in predictable anatomical locations at predictable developmental times, hence its name «programmed» cell death. Morphologically,

the cells dying in the embryo appear apoptotic, although the molecular mechanism of apoptosis in the embryonic cells may differ from that in the adult cells used in the more commonly studied models of apoptosis, in which cell death is induced pharmacologically (reviewed in Parchment, 1991).

Programmed cell death first occurs during embryogenesis in the ICM during blastulation (El-Shershaby and Hinchliffe, 1974, 1975; Handyside and Hunter, 1986; Pierce *et al.*, 1989b). At the mid- to late-blastocyst stage, a few isolated apoptotic cells appear in the midst of neighboring, viable cells. These dying cells show the morphology of apoptosis: condensed chromatin, fragmenting nuclei, and phagocytosis by adjacent cells. The apoptosis is specific and programmed developmentally, because only a few ICM cells die and all blastocysts contain apoptotic cells. It has been estimated that 6-8 ICM cells, or about 10% of the blastocyst's cell mass, is lost via programmed cell death.

Concomitant with the appearance of apoptotic cells, the ICM loses its potential to regenerate trophectoderm (Handyside, 1978; Hogan and Tilly, 1978, 1981; Pierce *et al.*, 1988, 1989b). ICMs from both early and late blastocysts can be isolated by destroying the trophectodermal wall. ICMs isolated from blastocysts at early stages of blastulation regenerate a trophectodermal layer within 24-36 h and re-expand with fluid, thereby producing a smaller yet structurally correct blastocyst. In contrast, ICMs from blastocysts at late stages of blastulation do not regenerate trophectoderm, and some may even form two-layered embryoid bodies, indicating the expression of endoderm which overlays the ICM.

With these data in hand, we proposed that the death of ECa 247 cells was due to the same mechanism that induces programmed cell death of the pre-trophectodermal ICM cells (Pierce *et al.*, 1989b). This mechanism purges the ICM of cells with trophectodermal potential before germ layer formation, perhaps preventing ectopic trophectoderm. The ECa 247 cells were being induced to die because they express this same developmental potential in the blastocyst — ECa 247 cells localize predominantly to the trophectodermal wall (Pierce *et al.*, 1987) and do not form chimerae (Pierce *et al.*, 1989b), although they are regulated by the blastocyst (Pierce *et al.*, 1984). The mechanism of cell death exhibited the expected specificity, as P19 cells, which localize to the ICM after incorporation into the blastocyst and caricature cells destined to form non-trophectodermal tissues, do not die (Pierce *et al.*, 1989b). Thus, the caricature principle (Pierce and Speers, 1988) extended into the embryo, showing that cancer cells can serve as probes of normal embryonic mechanisms (Parchment and Pierce, 1989), if their phenotype is appropriately chosen (Pierce *et al.*, 1989b). Perhaps more importantly we had an *in vivo* model system for attempting to identify the mechanism causing programmed cell death in the embryo.

These results increased our understanding of embryonic regulation of cancer as well (Pierce *et al.*, 1989a, 1990; Parchment *et al.*, 1990a). Suppression of tumor formation is thus due to the combined effects of induced apoptosis and abrogation of the malignant phenotype. At this point, it seemed likely that the regulation of B-16 melanoma cells by the embryonic limb may in part be due to cell death, depending upon the anatomic site of implantation along the limb bud, because programmed cell death between the digits and between the newly-forming bones is extensive at day-14 (Parchment and Pierce, 1989). We thus interpreted the death of the B-16 cells in the limb bud-conditioned medium as a biologically relevant phenomenon. With this *in vitro* model in hand, we set out to identify the mechanism inducing programmed cell death in the embryo.

Identification of H_2O_2 generated by amine oxidase activity as the cause of programmed cell death in the blastocyst: death due to murder rather than suicide

We used the *in vitro* model system with B-16 cells to pursue the purification of the cytotoxic activity in the limb bud-conditioned medium (Parchment and Pierce, 1989). We eventually switched to homogenates of day-14 limb buds as starting material for purification, because these contained a higher concentration of activity than conditioned media. We found that the activity was <1,000 Da in size, bound strongly to cation exchange resins, and co-eluted from cation exchange HPLC columns with two polyamine standards (spermidine and spermine). No activity was found in the macromolecular fraction of the extract. These results implied that the limb bud cells released spermine and spermidine into the extracellular space where they were oxidized by serum amine oxidases derived from the serum supplement of the culture medium. Oxidation of polyamines by amine oxidases generates H_2O_2 and cytotoxic aminoaldehydes and acrolein, all of which are cytotoxic (Gahl and Pitot, 1978; Swanson and Gibbs, 1980; Patt *et al.*, 1982; Ali-Osman and Maurer, 1983). H_2O_2 and probably acrolein can induce apoptosis in other systems (Parchment, 1991).

In confirmation of our conclusion that the *in vitro* toxicity of the limb bud extract or conditioned medium was due to polyamine oxidation, we were able to show that limb bud extracts were not toxic in serum-free medium or in the presence of serum containing aminoguanidine, an inhibitor of serum amine oxidase (8). At that time, these results were very disappointing, because they suggested that we had simply purified the source of artefactual *in vitro* toxicity that had plagued the chalone scientists of thirty years ago (for example, Byrd *et al.*, 1977; Rijke and Ballieux, 1978; Dewey, 1980).

We thus temporarily abandoned the *in vitro* model system with B-16 cells and turned our attention to a second model system from which to isolate the inducer of programmed cell death in blastocoele fluid (Parchment *et al.*, 1990b). This system was an embryonal carcinoma caricature of the late blastocyst. The ascites form of the embryonal carcinoma C44, derived from OTT6050, forms myriads of small bodies in the peritoneal cavity as an ascites tumor. Although these small bodies resembled blastocysts by light microscopy (Fig. 2A), an electron microscopic examination revealed that they are composed of an outer layer of primitive endoderm covering an inner mass of embryonal carcinoma cells (Fig. 2B and C). Apoptotic cells were not infrequent (Fig. 2D). When placed into culture for three days, these embryoid bodies expand with fluid, which we suspected would correspond to the blastocoele fluid of late-stage blastocysts. We were excited to find that the environment of this fluid-filled cavity suppressed colony formation by ECa 247 cells but not P19 cells. A technique was then developed to isolate the blastocoele-like fluid from these cysts without damaging the cells and therefore without contaminating the fluid with intracellular substances. To our delight, we found that this isolated fluid was preferentially toxic to ECa 247 over P19 *in vitro*. Furthermore, there were concentrations of blastocoele-like fluid which killed 50% of ECa 247 cells but 0% of P19 cells. This isolated C44 fluid contained a cytotoxic activity that exhibited the same specificity as giant blastocoele fluid.

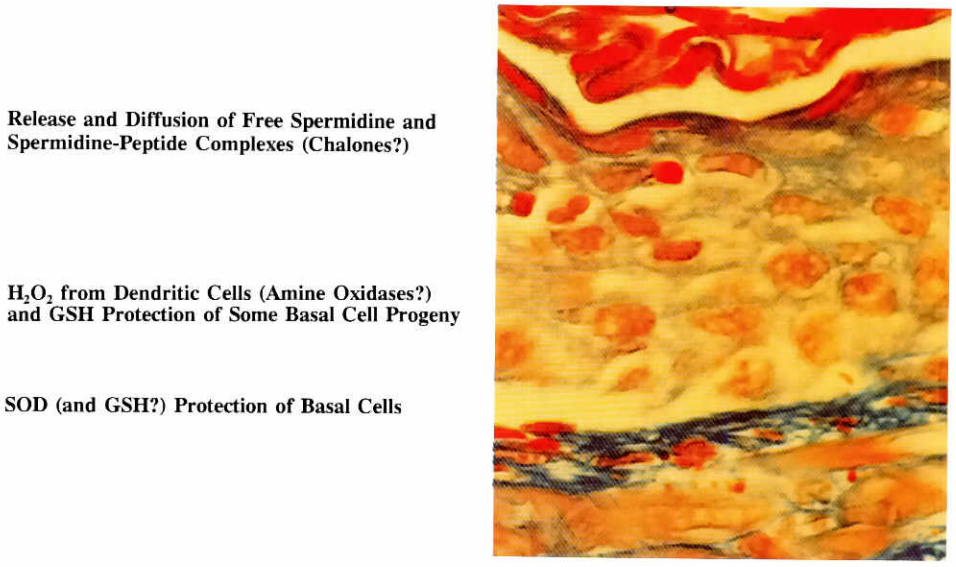

Fig. 3. A hypothetical model of regulation of tissue renewal in the epidermis based upon the known roles of polyamines and free-radicals in regulating the ICM of the blastocyst.

We thus pursued the purification of this activity from C44 fluid (Gramzinski *et al.*, 1990). To our surprise, this activity co-purified with spermine and spermidine, just like the activity from limb buds or limb bud-conditioned medium (Parchment and Pierce, 1989). All of the preferential toxicity of the C44 fluid *in vitro* was due to its small molecular weight fraction, and was completely inhibited by co-addition of aminoguanidine to the culture medium to inhibit serum amine oxidase. Serum amine oxidase substrates and even H_2O_2 added directly to the bioassays could mimic the preferential toxicity of C44 fluid for ECa 247 cells, and catalase could prevent the toxicity of the C44 fluid, indicating that the toxicity was due to the H_2O_2 rather than the aldehydes. Furthermore, chemical analysis of the C44 fluid showed the presence of several polyamines which are substrates for several amine oxidases, including tissue oxidases.

We spent many an afternoon wondering why we were always finishing with polyamines when we had started with such diverse model systems, one of which seemed to exactly model the blastocoele environment. Two independent approaches had indicated that H_2O_2 from polyamine oxidation was the inducer of programmed cell death in the embryo. We often discussed whether this mechanism, concluded to be artefact and abandoned as physiologically-irrelevant years before, could actually be the *in vivo* mechanism. Finally, we arrived at some logic to untangle this conundrum. We reasoned that had we isolated any biochemical other than polyamines as the inducer of cell death, we would readily have concluded that it was active in the embryo. It then seemed completely illogical to refrain from concluding that polyamine oxidation caused programmed cell death *in vivo*, without at least an attempt at disproof. We were being biased by the «negative publicity» of this mechanism from many years ago. We decided to be faithful to our data and conclusions therefrom, and set out to challenge this hypothesis directly.

We decided to test three predictions of our hypothesis that H_2O_2 from amine oxidase-dependent polyamine oxidation causes programmed cell death in the embryo. Any of these tests had the potential to disprove the hypothesis. The first test was based upon the reasoning that catalase should prevent the death of ECa 247 cells exposed to the blastocoele fluid of giant blastocysts, if extracellular H_2O_2 is in fact mediating cell death as the hypothesis predicts. To this end, ECa 247 cells in *zonae* carriers were placed into the blastocoele cavity of giant blastocysts with and without co-injection of catalase (Pierce *et al.*, 1991). Catalase significantly decreased the death rate of the ECa 247 cells from 35% to 14%. The presence of a few dying cells (14%) in the presence of catalase suggests either the need for higher catalase levels or a small contribution by toxic aldehydes to the cell death.

The second test was based upon the idea that we could disprove the hypothesis if the C44 embryoid bodies and day-14 limb buds did not contain any amine oxidase activity, because the activity *in vitro* depended upon an active amine oxidase. The results of the first test had proved that extracellular H_2O_2 played a major role in inducing cell death *in situ*, but it did not reveal the identity of the H_2O_2-generating system, and several enzyme systems are known to generate H_2O_2. Although we cannot prove at this time that amine oxidases are the source of extracellular H_2O_2 in the embryo, they cannot be excluded either, because a polyamine oxidase that can oxidize spermine and spermidine and some derivatives was detected in the day-14 limb bud and the blastocoele-like fluid from C44 (Parchment and Pierce, 1989; Parchment, 1991). The pH optimum and inhibitor specificities of this polyamine oxidase indicate a similarity to the polyamine oxidase in liver of adult rodents (Parchment, 1991). No other amine oxidase types were detected in either source, including diamine oxidase and plasma (serum) amine oxidases.

The third test was based upon the idea that normal ICM cells from early blastocysts should be similarly affected by exposure to blastocoele fluid in giant blastocysts: ICMs from early blastocysts should lose their capacity for regenerating trophectoderm and dead cells should be evident as a result of exposure to this fluid. To test this, ICM cells from early blastocysts were therefore placed in *zonae pellucidae* carriers and inserted into the cavity of giant blastocysts

(Pierce *et al.*, 1989b). After 24 h of exposure, ICMs were found to contain apoptotic cells and these exposed ICMs were unable to regenerate a trophectodermal layer; instead they produced two-layered embryoid bodies characteristic of late-stage ICMs. In contrast, no apoptotic cells could be found in the ICMs exposed to cell culture medium in *zonae* carriers; these specimens were able to regenerate trophectoderm and reform blastocysts.

Thus, three attempts to disprove the hypothesis have failed. Current research is focused upon the development of model systems to allow controlled exposure to inhibitors of different amine oxidases to test the prediction that programmed cell death will not occur when amine oxidase activity is inhibited.

Conclusions and implications for tissue renewal *in vivo* and *in vitro*

In summary these data suggest a heretofore unsuspected relationship between programmed cell death, extracellular polyamine oxidation and H_2O_2, mechanisms of free radical-induced cell death, and the regulation of the phenotypic composition of embryonic tissue. We have concluded that extracellular H_2O_2 from polyamine oxidation causes the programmed cell death of ICM cells with trophectodermal potential and their malignant caricatures (Pierce *et al.*, 1989b). This purging of pre-trophectodermal cells from the ICM is an irreversible change in phenotypic composition, and it is thus an example of histogenetic programmed cell death (Parchment, 1991). It makes good sense why these cells should be eliminated from the ICM at this stage, for it would seem deleterious to retain trophectodermal potential within the cell mass that will form the germ layers because of the potential for ectopic trophectoderm. Whether this mechanism also causes morphogenetic programmed cell death in the embryo, in which many more cells die, often in large groups, remains to be determined. However, the release of polyamines into the extracellular space by cultured limb bud cells suggests that the same mechanism may also cause the separation of digits and bone anlagen in the developing limb bud (Parchment and Pierce, 1989).

One may ask why the discrepancy between the complete loss of trophectodermal potential from the ICM (Handyside, 1978; Hogan and Tilly, 1978, 1981; Pierce *et al.*, 1988) and the death of only 42% of the ECa 247 cells (Pierce *et al.*, 1989b). It is likely that this discrepancy results from technical rather than biological causes. In fact, 15% of the early ICMs exposed to blastocoele fluid retained the capacity for regenerating the trophectodermal layer (Pierce *et al.*, 1989b), which is much higher than the expected 0% frequency of pre-trophectodermal ICM cells surviving in the intact blastocyst. The dose-response curve for ECa 247 cell death induced by H_2O_2 is so steep (a drop in concentration from 20 to 10 μM decreases the frequency of cell death from 100% to 5%) (Gramzinski *et al.*, 1990), that any slight dilution of the H_2O_2 in the blastocoele fluid during blastocyst manipulation, or slightly lower H_2O_2 concentrations in blastocoele fluid of giant blastocysts, could dramatically lower the rate of cell death. A second explanation may have biological relevance. Apoptotic ICM cells have been reported within the ICM of the intact blastocyst (El-Shershaby and Hinchliffe, 1974, 1975; Handyside and Hunter, 1986), yet we were measuring the cell death after exposure to blastocoele fluid, which is outside the ICM. If the H_2O_2 were generated predominantly by polar trophectoderm, then there would be a gradient of H_2O_2 concentration, with highest levels in the ICM. The rate of cell death of a pre-trophectodermal population would be less in the blastocoele cavity than in the ICM. These considerations indicate that conclusions from results with these model systems are only qualitative, not quantitative.

Perhaps the most surprising result from our research was the finding that extracellular H_2O_2 performs a critical function in the embryo in controlling tissue composition and mass. This paracrine regulatory function of H_2O_2 contrasts sharply with its historical associations with only pathogenetic mechanisms, including necrosis. However, extracellular H_2O_2 may induce apoptosis by inactivating plasma membrane Ca^{2+}-ATPases, thereby increasing cytosolic calcium for prolonged periods of time, and perhaps activating the Ca^{2+}, Mg^{2+}-endonuclease (Parchment, 1991). Other investigators have shown that low concentrations of intracellular H_2O_2 cause apoptosis, whereas high levels cause necrosis (Parchment, 1991). The concentration in the blastocoele fluid did not cause immediate cell death, and in fact more than 10 h were required to detect apoptotic cells by microscopic examination, although a «point of no return», characteristic of embryonic cells destined to die (Saunders, 1966), may have been reached earlier. This delay in cell death suggests that the H_2O_2 concentration in blastocoele fluid is equivalent to the low concentrations reported to cause apoptosis rather than necrosis. However, higher levels sufficient to cause necrosis may be present during other embryonic events, such as the massive zonal cell death that occurs during limb morphogenesis (Saunders, 1966; Parchment, 1991).

Finding extracellular H_2O_2 to be the cause of programmed cell death means that the cell death is «murder» in contrast to the traditional view that it is «suicide» or «death from within» (Parchment, 1991). Given that presumably all ICM cells are exposed to the extracellular H_2O_2, why do only the 10% that are pre-trophectodermal cells die? An equivalent question is how can this mechanism cause the death of ECa 247 but not P19? This is a traditional curiosity of those who study apoptosis: what is the molecular basis for the highly specific nature of cell death, allowing one cell to be killed, or to kill itself, without affecting any of the neighboring viable cells? In this regard, P19 cells are killed *in vitro* by C44 fluid, but much higher concentrations are required; they are likewise much more resistant to H_2O_2 cytotoxicity (Gramzinski *et al.*, 1990). This increased resistance is due to a glutathione-dependent mechanism that is absent in ECa 247 cells (Gramzinski *et al.*, 1990). This glutathione-dependent mechanism must also be responsible for P19's resistance to apoptosis in the blastocyst, because P19 cells previously depleted of intracellular glutathione become sensitive to the H_2O_2 in the blastocoele fluid (Pierce *et al.*, 1991). Embryologically, this result suggests that the induction of the non-trophectodermal lineage in the ICM includes the expression of free radical protective mechanisms, or conversely, that induction of pre-trophectodermal potential includes the repression of these protective mechanisms. It is interesting that lineage specificity involves resistance to free radical damage. Inappropriate expression of these protective mechanisms may be responsible for the death of all of the ICM cells in some embryos, leading to the spontaneous abortion of a «membranes-only» conceptus.

Polyamines are ubiquitously involved in control of differentiation and proliferation in renewing tissues, including the embryo, but their exact mechanisms of action have been difficult to pinpoint (Boynton *et al.*, 1980; Heby, 1981; Schindler *et al.*, 1983; Oredsson *et al.*, 1985; Pegg and McCann, 1988). The data from our studies suggest a novel function for polyamines: non-toxic storehouses of H_2O_2 equivalents. Although functions for reaction products from polyamine

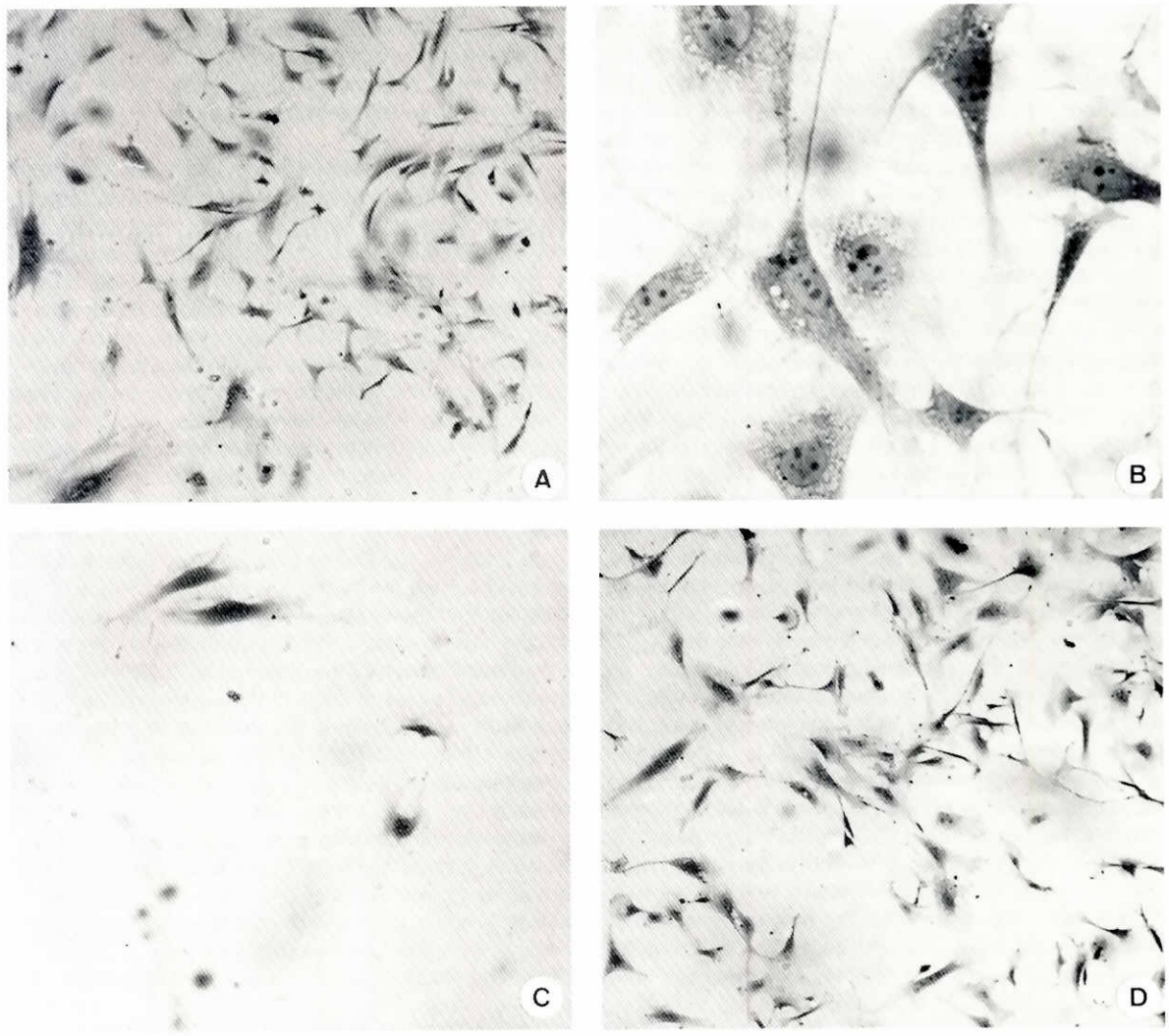

Fig. 4. Amine oxidase-dependent polyamine oxidation causes the crisis of spontaneous transformation. (A) *A pre-crisis (passage 2) culture of mouse embryo cells from day-14 limb buds.* **(B)** *As these cultures enter the crisis phase, the cells become vacuolated and cease dividing.* **(C)** *Crisis leaves only rare survivors from the original culture.* **(D)** *Crisis has been prevented in this mouse embryo cell culture beyond passage 8 by adding an inhibitor of serum amine oxidases to the culture medium (aminoguanidine).*

metabolism have sporadically appeared in the literature, the results in the embryonic models provide the first proof of a physiologic function for the H_2O_2 metabolite of polyamine catabolism.

From the perspective of regulation of tissue mass in renewing tissues, H_2O_2 exposure in the blastocyst reduces the mass of the ICM by 10% (El-Shershaby and Hinchliffe, 1974, 1975; Handyside and Hunter, 1986). In addition, our early work showed that cell death of some but not all cells of a population is equivalent biologically to inhibition of proliferation (Parchment and Pierce, 1989; Pierce *et al.*, 1990; Parchment, 1991). Given these results, is it conceivable that apoptosis in renewing epithelia of the adult is caused by H_2O_2 from polyamine oxidation and that it thereby regulates the mass of the renewing tissue (Parchment *et al.*, 1990a; Pierce *et al.*, 1990), a function once proposed for chalones? In the epidermis, apoptotic cells are evident not only in the corneum as a result of terminal differentiation but also in the cell layer immediately superficial to the basal cell layer. Polyamines, amine oxidases, free radical detoxification systems, and glutathione peroxidases are present, and DFMO influences cell proliferation (Perchellet *et al.*, 1986, 1987; Carraro and Pathak, 1988; Reiners *et al.*, 1990; Koza *et al.*, 1991). Dendritic cells that influence this suprabasal layer were recently shown to generate H_2O_2 (Ledger *et al.*, 1991). From our work on programmed cell death in the embryo, it can be proposed that this H_2O_2 is generated by amine oxidases associated with the dendritic cells (Fig. 3). Furthermore, such a model predicts that the H_2O_2 in these dendritic cells is responsible for all of the apoptosis that occurs in some but not all of the basal cell progeny in this layer. The cells that survive exposure to this

environment would do so because of glutathione-dependent or catalase-dependent protective mechanisms (Perchellet *et al.*, 1986; Reiners *et al.*, 1990). It seems possible that basal cells may even produce two lineages of progeny, only one of which is usually used for keratinization but the second of which can be deployed under specific physiological or pathological situations. Basal cells are extremely well protected from free-radicals, perhaps even better than their progeny, as evidenced by the restriction of superoxide dismutase expression to just the basal layer (Kobayashi *et al.*, 1991). It is interesting that a relationship between superoxide dismutase and keratinocyte proliferation has been proposed (Ohkuma *et al.*, 1987).

How would the polyamine oxidation mechanism regulate renewal of the epidermis? Terminally differentiating keratinocytes would release polyamines, especially spermidine (Martinet *et al.*, 1990; Piacentini *et al.*, 1990), into the extracellular space as they differentiate, where they can be conjugated to cell surface proteins by transglutaminase, the levels of which increase as part of the apoptosis program (Parchment, 1991; Martinet *et al.*, 1990; Piacentini *et al.*, 1988, 1990). However, some of these polyamines likely escape conjugation, or are released by proteolysis, and diffuse through the epidermal layers. Greater numbers of keratinizing cells would produce more polyamines for amine oxidase-dependent generation of H_2O_2 in the dendritic cells and increase the death rate of basal cell progeny in the suprabasal layer. Conversely, an insufficient mass of keratinizing cells would decrease the amount of H_2O_2 generated by dendritic cells in the suprabasal layer, and permit a greater fraction of basal cell progeny to survive and differentiate, increasing epidermal mass. In this regard, it is intriguing that psoriatic lesions contained elevated levels of conjugated polyamines (Martinet *et al.*, 1990); in the proposed model this would result in decreased levels of free polyamines in the dendritic layer, decreased H_2O_2, and increased and superfluous keratinocyte production. It is also intriguing that the spermidine content of keratinocytes dramatically falls during Ca^{2+}-induced differentiation, but putrescine content falls less dramatically, and spermine content does not fall at all (Piacentini *et al.*, 1990), indicating precise regulation of polyamine release and conjugation in the different layers of the epidermis. Our hypothesis (Fig. 3) can also accommodate regulation of amine oxidase activity in the dendritic cells; down-modulation would allow more polyamines to diffuse past the dendritic cell layer without oxidation into the basal layer, where they could stimulate basal cell proliferation.

As the reader may recognize, this model (Fig. 3) essentially proposes that the H_2O_2-polyamine system exhibits many of the characteristics expected of chalones in renewing tissues of the adult, such as epithelia. It regulates tissue mass via a feedback mechanism between the differentiated and renewing compartments and balances cell loss via cell death with cell gain via proliferation. However, there is a subtle point: neither the H_2O_2 nor the polyamines *per se* are the chalones, because polyamines are the diffusible substance but only the storehouse for the active principle, yet H_2O_2 is the chalone which is not diffusible (because of its reactivity) and which is generated *in situ* only at the site where tissue mass is regulated (progenitor level). If the epidermal chalone is H_2O_2 from polyamine oxidation, one can easily understand why it has been so difficult to purify: H_2O_2 is too reactive to purify and too non-specific to assay when removed from the architecture of the epidermis; polyamines are only the precursor for the chalone and are, therefore, inactive *in vitro* in chalone bioassays. The investigators of many years ago that isolated chalones «contaminated» by polyamines (Rijke and Ballieux, 1978; Dewey, 1980) may have been closer to the truth than any of their numerous critics realized. This hypothesis of the role of polyamines in regulating tissue renewal and tissue mass offers several testable predictions which we are currently examining.

The research described herein has led not only to the identification of a second embryonic mechanism that contributes to suppression of malignancy but also to a novel model of programmed cell death in the embryo which may turn out to have implications for understanding tissue renewal in the adult. The data show that the specificity of apoptosis in the embryo is due to the nature of the exposed cell rather than the nature of the signal, and that programmed cell death is murder rather than suicide. The presence of apoptotic cells in the midst of viable neighboring cells has previously been taken as evidence for cellular suicide (Parchment, 1991), and theoretical mechanisms have been proposed to explain this «death from within». However, the results from the research on programmed cell death in the blastocyst are not consistent with this view.

In addition, the finding that limb bud cells release polyamines into cell culture medium (Parchment and Pierce, 1989) led us to discover that their oxidation by serum amine oxidases causes the crisis of spontaneous transformation (Fig. 4), yet not spontaneous transformation itself (Parchment *et al.*, 1990c). The 4-6 week delay in the appearance of crisis in mouse embryo cell cultures may be due to the gradual fall in the free radical protective mechanisms over time that are initially high, because they were protecting these cells from the programmed cell death occurring in the tissue from whence they came. These data on crisis show that polyamine oxidation products can also regulate long-term tissue renewal *in vitro*, probably because the high extracellular polyamines released by the predominant, non-renewable cell types selectively kill the renewing cell population within days of culture initiation (Parchment and Natarajan, 1993; unpublished data) and so the cultures are destined to expire over time. The embryonic studies also led to the discovery that 2-mercaptoethanol protects cultured cells from serum amine oxidase-dependent polyamine toxicity (Parchment and Pierce, 1989; Gramzinski *et al.*, 1990), which then led to the finding that it can also prevent the crisis of spontaneous transformation (Parchment *et al.*, 1990c). There are many significant implications of this discovery for cell culture technology, because of the many *in vitro* systems in which performance is enhanced by low oxygen tension or the addition of 2-mercaptoethanol (Parchment and Natarajan, 1993).

Dr. Pierce has a reputation for discovering biological principles with wide-ranging implications, and the research described in this paper exemplifies the reason for this reputation.

The original observation that extracellular substances in the embryo kill cancer cells (Parchment and Pierce, 1989; Pierce *et al.*, 1989b) has impacted on our understanding of biological phenomena as diverse as the embryonic control of cancer, programmed cell death, polyamine regulation of tissue mass and renewal and the crisis of spontaneous transformation. This diverse impact is certainly a fitting tribute to Dr. Pierce and his intuition about the workings of biological systems.

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