Apoptosis in Metanephric Development

Chizuko Koseki,* Doris Herzlinger,‡ and Qais Al-Awqati

Departments of Medicine and Physiology, College of Physicians and Surgeons of Columbia University, New York, New York 10032

Abstract. During metanephric development, non-polarized mesenchymal cells are induced to form the epithelial structures of the nephron following interaction with extracellular matrix proteins and factors produced by the inducing tissue, ureteric bud. This induction can occur in a transfilter organ culture system where it can also be produced by heterologous cells such as the embryonic spinal cord. We found that when embryonic mesenchyme was induced in vitro and in vivo, many of the cells surrounding the new epithelium showed morphological evidence of programmed cell death (apoptosis) such as condensed nuclei, fragmented cytoplasm, and cell shrinking. A biochemical correlate of apoptosis is the transcriptional activation of a calcium-sensitive endonuclease. Indeed, DNA isolated from uninduced mesenchyme showed progressive degradation, a process that was prevented by treatment with actinomycin-D or cycloheximide and by buffering intracellular calcium. These results demonstrate that the metanephric mesenchyme is programmed for apoptosis.

Incubation of mesenchyme with a heterologous inducer, embryonic spinal cord prevented this DNA degradation. To investigate the mechanism by which inducers prevented apoptosis we tested the effects of protein kinase C modulators on this process. Phorbol esters mimicked the effects of the inducer and staurosporine, an inhibitor of this protein kinase, prevented the effect of the inducer. EGF also prevented DNA degradation but did not lead to differentiation. These results demonstrate that conversion of mesenchyme to epithelia requires at least two steps, rescue of the mesenchyme from apoptosis and induction of differentiation.

CONVERSION of mesenchyme to epithelia during kidney organogenesis occurs when the ureteric bud induces the mesenchyme to differentiate, a process that can be recapitulated in vitro using a transfilter organ culture system (7, 21). Invasion of the mesenchyme by the ureteric bud causes the appearance of epithelial cell markers such as type IV collagen, the alpha chain of laminin and uvomorulin, and the disappearance of mesenchymal proteins such as vimentin and N-CAM (4). Immediately following induction the cells undergo a burst of proliferation which raised the question of whether all the cells in the metanephric mesenchyme contributed to the formation of mature nephrons or whether only some of the cells were selected for differentiation. During morphogenesis of some tissues, cells are produced in large excess but rapidly die and only a few remain to form the mature tissue (5, 27).

Cell death occurs by two processes, necrosis or apoptosis whose cytological and biochemical characteristics are distinct (1, 5, 18, 25, 27). At the morphological level, necrosis is associated with early cell swelling and lysis of intracellular organelles but nuclear breakdown is a late event. On the other hand, in apoptosis (also termed programmed cell death

or cell suicide), nuclear fragmentation is observed early in the course and is associated with cytoplasmic shrinking and preservation of intracellular organelles. The cells eventually become fragmented and are phagocytosed by neighboring cells or by macrophages. In necrosis which is frequently associated with cell injury, the ATP levels fall early and protein synthesis is consequently inhibited. In apoptosis, ATP levels are frequently normal and protein synthesis is often increased. In fact, apoptosis can be prevented by inhibitors of RNA and protein synthesis. A characteristic finding is the early degradation of DNA to a size of 180–200 bp suggesting the activation of an endonuclease that digests DNA at the nucleosomes (26). This endonuclease is calcium activated (27).

Apoptosis plays an important role in morphogenesis of a variety of systems (5). It is responsible for clonal deletion in thymic development and maturation (15). Apoptosis allows the formation of digits when the interdigital cells die. In neural development it determines the correct relation between the size of sensory neural area and that of its target. In worm and insect development, several genes have been found whose disruption prevents apoptosis, one of which appears to contain a calcium-binding motif (5). The study of apoptosis has emphasized its role in the development of specific organs or tissues, but recent evidence has suggested a more generalized role (18). A gene was recently identified in *Caenorhabditis elegans*, *ced-9* whose expression was found

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Dr. Koseki's permanent address is National Institute of Cardiovascular Research, Osaka, Japan.

Dr. Herzlinger's present address is Department of Physiology and Biophysics, Cornell Medical College, New York, NY.

to prevent cell death in a variety of lineages suggesting that many cell types can constitutively progress towards apoptosis but need a factor that will rescue them from this fate (8).

While performing studies on the lineage of cells during metanephric development (9), we noticed that the cells surrounding the induced mesenchyme showed the morphological and biochemical characteristics of programmed cell death. An examination of the mechanism by which apoptosis was prevented in the selected cells suggests that activation of protein kinase C and possibly tyrosine kinases by the inducer is required for this inhibition of apoptosis.

Materials and Methods

The transfilter culture of mesenchyme was performed as previously described (11). Embryonic kidney and spinal cord were microdissected from gestation day 13 rat embryos at which time the ureteric bud had just started to penetrate the mesenchyme. Metanephric mesenchyme was freed from the endogenous inducer (ureteric bud) and placed on a transfilter (Transwell, pore size 3 μm; Costar, Cambridge, UK) and cultured for 24 h in the presence or absence of heterologous inducer. The embryonic dorsal spinal cord was attached to the under surface of the filter. For microscopy, the tissue was fixed with 2% paraformaldehyde in phosphate buffered saline and embedded in HistoResin (Kulzer, Heidelberg, Germany). 4-μm sections were stained with hematoxylin and eosin.

Measurement of DNA Degradation

The mesenchyme was microdissected from E13 embryos, isolated from ureteric bud and incubated with 25 μCi [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) in DME containing 10% FCS for 2 h at 37°C. The explants were then transferred onto the transfilter system and incubated in the absence of isotope for an additional 4–6 h in the presence or absence of added reagents. Before DNA extraction, the kidney mesenchyme was first dissociated by 5% trypsin in PBS for 30 min at 37°C. DNA was extracted as described (16) except that proteinase K treatment and phenol extraction were repeated twice. Extracted DNA was suspended in 15 μl tris-EDTA buffer and separated in 0.6% agarose gel containing ethidium bromide. The agarose gel was cut into 12 4-mm slices. Each slice was extracted in scintillation medium (Ready protein, Beckman Instruments, Palo Alto, CA) and radioactivity was measured. High molecular weight DNA is defined as the top three fractions of the agarose gel encompassing DNA fragments >5 kb and is expressed as a fraction of the total counts in the gel.

All assays were performed in a paired manner where each assay point used kidneys from three embryos. Each point was repeated three to seven times as noted in the text. In the experiments where the effects of the inducer was tested, only the dorsal spinal cord was used as an inducer since it was difficult to obtain enough quantities of the ureteric bud. In some experiments the dorsal embryonic spinal cord was cut into small pieces and incubated around the rudiments while in others a homogenate of the spinal cord was used. Spinal cord was homogenized by ten strokes in a glass/glass homogenizer in 10% FCS in DME. Examination of the homogenate revealed that it contained isolated viable cells as well as cell fragments. After the appropriate incubation period, the mesenchyme was frozen at −70°C until the DNA was extracted.

The final concentration of the various added reagents was, 5 μg/ml actinomycin-D (Sigma Chemical Co., St. Louis, MO); 10 μM cycloheximide (Sigma Chemical Co.); 20 μM BAPTA-AM (Molecular Probes Inc., Eugene, OR); 20 μM PMA (Sigma Chemical Co.); and 0.1 μM staurosporin (Calbiochem-Behring Corp., San Diego, CA).

Since each set of experiments was performed as a pair where fragments of metanephric mesenchyme were taken from the same animals and divided into two (or more) parts to examine the effect of additives, statistics were performed using the paired t-test where n refers to the number of experiments performed.

Results

When metanephric mesenchyme was isolated at embryonic day 13 (E13) from rat embryos and studied in a transfilter assay, the uninduced mesenchyme showed all the morphological characteristics of apoptosis including cell and nuclear fragmentation and condensed nuclei, (Fig. 1 A). While the induced mesenchyme converted to an epithelium (Fig. 1 B), it remained surrounded by cells that showed apoptotic changes. The experiment of Fig. 1 was performed 4–6 h after setting up the assay, hence the morphology does not show advanced tubulogenesis. It takes at least 24–48 h to have recognizable tubules.
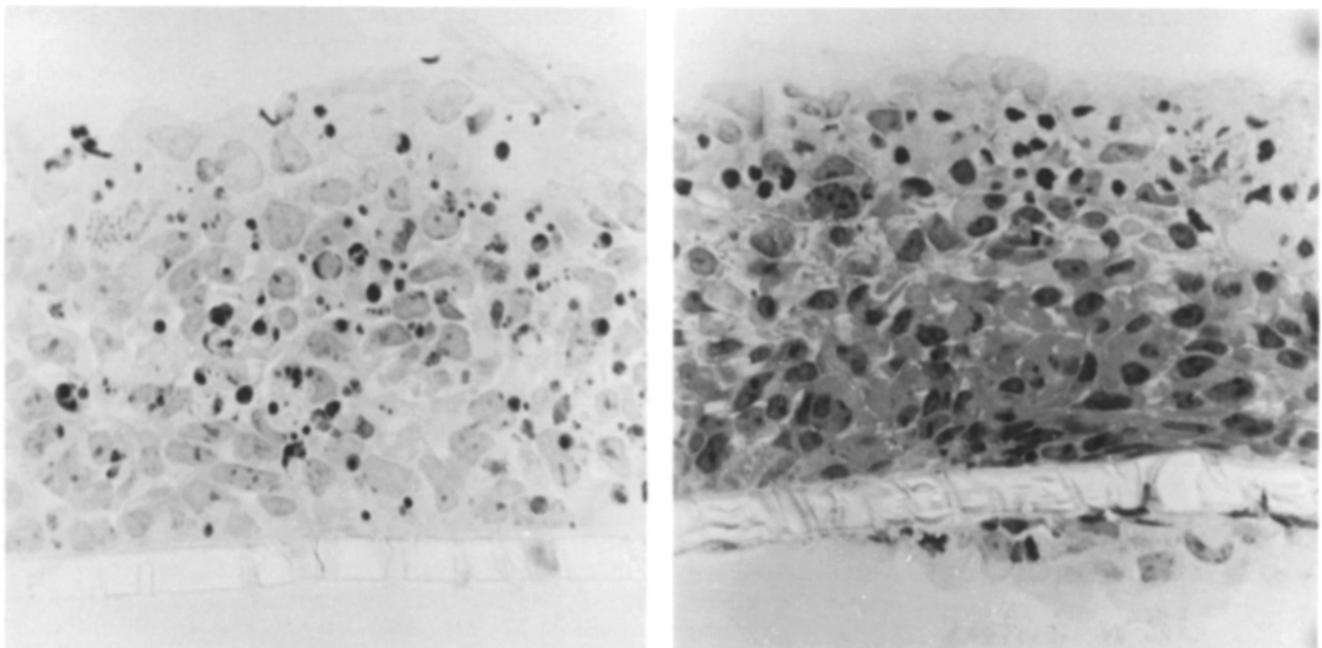

Figure 1. Transfilter culture of metanephric mesenchyme in the presence (*right*) and absence (*left*) of embryonic spinal cord. Note that most nuclei in the left panel are small, dense, and fragmented when compared to the nuclei of the induced sample. Note also that nuclear fragmentation is present in cells that surround the induced mesenchyme in the right panel as well.
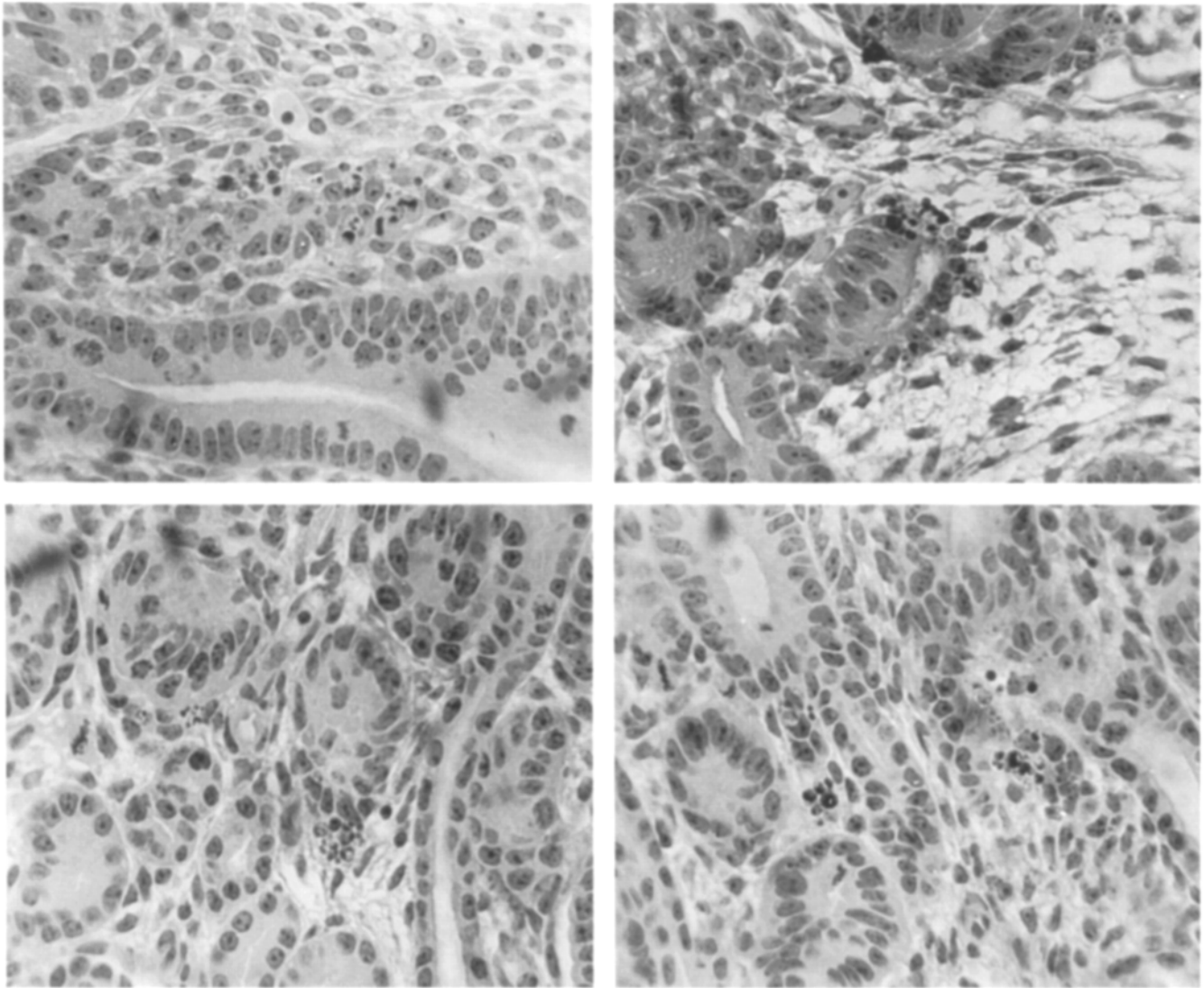
Figure 2. Presence of apoptosis in developing kidneys. Sections through E14 (upper left), E15 (upper right) and E19 (bottom panels) embryonic kidneys. Note the groups of apoptotic cells outside the developing tubules.

Examination of sections of embryonic kidney isolated from later stages of development (E14 and E19 embryos) showed that there were islands of apoptotic cells between developing nephrons (Fig. 2). These results demonstrate that apoptosis is a prominent feature of metanephric development and is not an artifact of the in vitro transfilter assay system.

One of the characteristics of apoptosis is the synthesis of a calcium-sensitive endonuclease (26, 27). To test for activation of this enzyme in the metanephric mesenchyme we incubated E13 mesenchyme in a transfilter chamber with [³H]thymidine for 2 h and isolated DNA at different times of culture and analyzed it by agarose gel electrophoresis. DNA was degraded rapidly such that by 24 h all of the DNA was in low molecular weight form and by 48 h all of the DNA was about 200 bp in size (Fig. 3). High molecular weight DNA (>5 kb) was reduced to 35–45% of the total DNA within 6 h of incubation. When uninduced mesenchyme was incubated with the inhibitors of RNA synthesis (actinomycin D) or protein synthesis (cycloheximide), the fraction of DNA that was in high molecular weight increased to 74 and 81%,

respectively ($n = 5$ each; $P < 0.01$) (Fig. 4, *A* and *B*). The endonuclease of apoptosis is known to be activated by divalent cations. We buffered the intracellular calcium by incubating the mesenchyme in the permeant calcium chelator, BAPTA-AM and found that this also significantly prevented the degradation of DNA (Fig. 4 *C*). Inhibition of DNA degradation by these reagents did not lead to tubulogenesis as assayed morphologically (data not shown). These morphological and biochemical results demonstrate that the metanephric mesenchyme is programmed for apoptosis.

We also performed experiments to test for DNA degradation during kidney development in situ. Kidney rudiments were removed from E13-E19 embryos and labeled for 2 h with [³H]thymidine. The DNA was then extracted without further incubation and separated on agarose gels. As shown in Table I, there was DNA degradation in situ at all stages of kidney development which amounted to ~10% of the total DNA at E12-E14. At later stages of development, E15 and E19 there appeared to be an increase in the level of DNA degradation. That DNA degradation was demonstrated in situ, suggests that apoptosis is one of the physiological con-
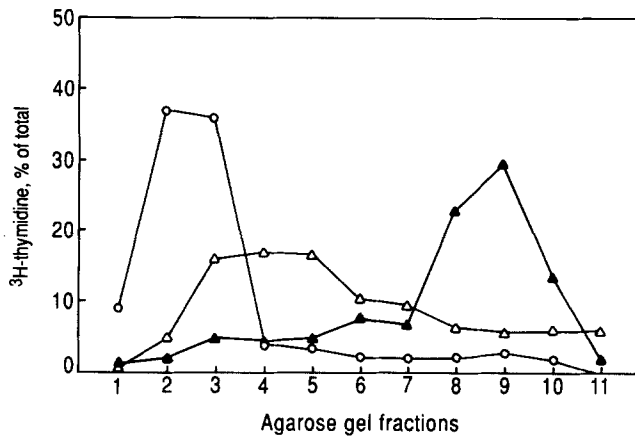
Figure 3. DNA degradation in mesenchymal cells. Metanephric rudiments were isolated from E13 embryos and labeled with [³H]-thymidine for 2 h and the DNA was immediately extracted and separated on agarose gels (O—O), or incubated for 24 (△—△), or 48 h (▲—▲) at 37°C in the absence of label before extraction. DNA is expressed as % of the total cpm. The total amount of ³H label present in the 24- and 48-h experiments was less than 25% of the initial label suggesting that by that time most of the DNA had been degraded to small molecular weight DNA and was lost either during extraction or during electrophoresis. The 21-kb marker is at the first or second slice and the 0.2 kb would be in slice 10-12.

Table I. Fraction of Total [³H]Thymidine-labeled DNA in High Molecular Form (Perccent HMWDNA)

DNA Degradaton In Situ

Gestation age	E12	E13	E14	E15	E19
Percent HMWDNA	92 ± 1	91 ± 2	91 ± 2	83 ± 5	67 ± 10
n	3	5	5	5	4

Kidneys were removed from gestation day 12 to 19 and incubated in [9H]thymidine for 2 h. DNA was extracted and separated on agarose gels. HMWDNA is defined as the top three fractions of the gel comprising DNA greater than 5 kb in molecular weight.

comitants of metanephric development and confirms the morphological findings of Fig. 2.

Conversion of kidney mesenchyme occurs when an outgrowth of the Wolffian duct, the ureteric bud, invades it and causes the appearance of epithelial markers (4, 21). In early experiments, Grobstein found that the process of mesenchymal conversion can occur in vitro in transfilter organ cultures and that it can also be produced by a heterologous inducer, embryonic spinal cord (6, 7). We tested the hypothesis that the inducer rescues the mesenchyme from apoptosis by studying DNA fragmentation. In all our studies we used the embryonic spinal cord since the dissection and isolation of
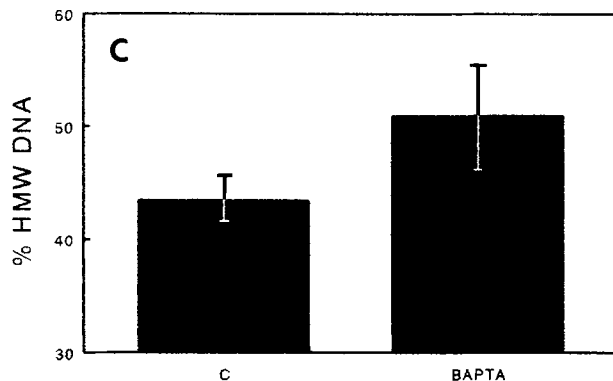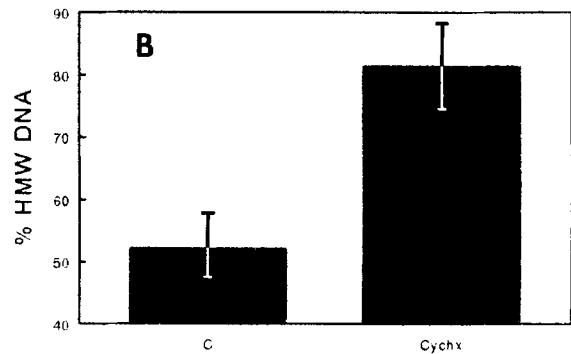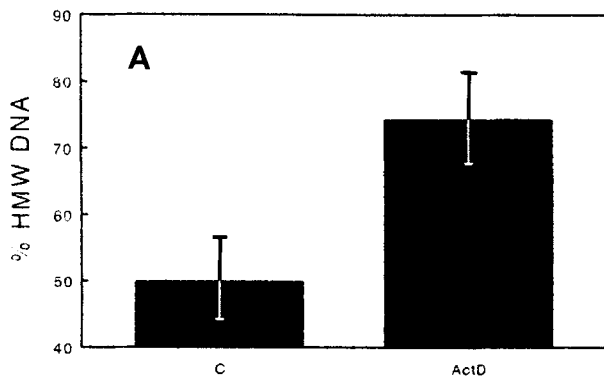

Figure 4. DNA degradation in mesenchymal cells. High molecular weight DNA (HMW DNA) as a fraction of total DNA is plotted for different experimental conditions. (A) Actinomycin D, 5 μg/ml; $n = 5$. (B) Cycloheximide, 10 μM, $n = 5$; (C) BAPTA-AM, 20 μM, $n = 5$; $P < 0.01$ for all additions compared to no additions.
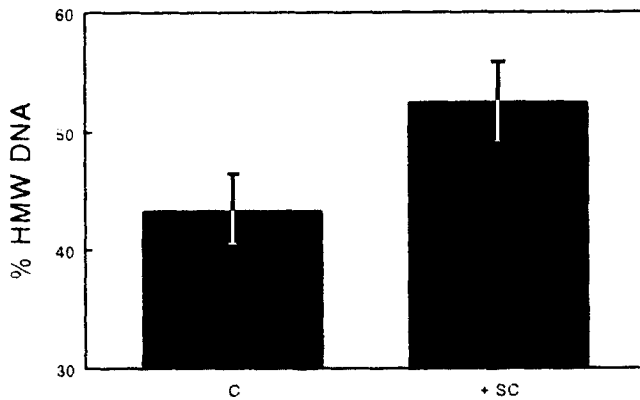
Figure 5. DNA degradation in mesenchymal cells cultured with embryonic spinal cord. (SC), $n = 7$, $P < 0.01$.

the ureteric bud proved difficult. We found that DNA fragmentation was prevented when mesenchyme was incubated with embryonic spinal cord, Fig. 5. The protection afforded by spinal cord was much weaker than the effect of inhibitors of protein and RNA synthesis. Reducing the amount of inducer at constant target mesenchyme size reduced the effect (data not shown).

Induction by the spinal cord could occur by either secreting a factor that activates the mesenchyme or by cell to cell contact. Both mechanisms act on the surface and hence there must be some kind of signal transduction mechanism to allow the inhibition of apoptosis. PMA, an activator of protein kinase C was found to inhibit apoptosis in lymphocytes (17), and it also reduced DNA degradation in metanephric mesenchyme (Fig. 6, top). PMA did not result in any additive effect when it was used with the spinal cord, Fig. 6 A suggesting that the inducer itself acts through this pathway. Stronger evidence was provided by the finding that staurosporin, an inhibitor of protein kinase C, prevented the protective effect of the inducer on DNA degradation (Fig. 6 B). Morphological observation of explants which were devoid of ureteric bud showed that phorbol esters did not cause the appearance of tubules (data not shown).

Recently, Weller et al. (24) studied the effect of a number of soluble growth factors on the conversion of mouse metanephric mesenchyme and found that none of a large number of factors including EGF were capable of inducing the conversion. However, EGF caused an increase in DNA synthesis. The metanephric rudiment produces TGF alpha (which binds to the EGF receptor) and antibodies to this growth factor prevent tubulogenesis in rat metanephros suggesting that this protein is necessary for renal development (20). We studied the effect of EGF on apoptosis in rat mesenchyme and found that it prevented the degradation of DNA that occurs in the absence of the inducer, Fig. 7. These results imply that prevention of apoptosis can be achieved by activation of protein kinase C or a tyrosine kinase. Since tyrosine kinases frequently constitute the cytoplasmic portion of receptors for several growth factors and hormones while protein kinase C is usually downstream from plasma membrane receptors, the present results suggest that such a cascade might be involved in the inhibition of apoptosis.

Discussion

Cell death occurs by two mechanisms, apoptosis and necro-
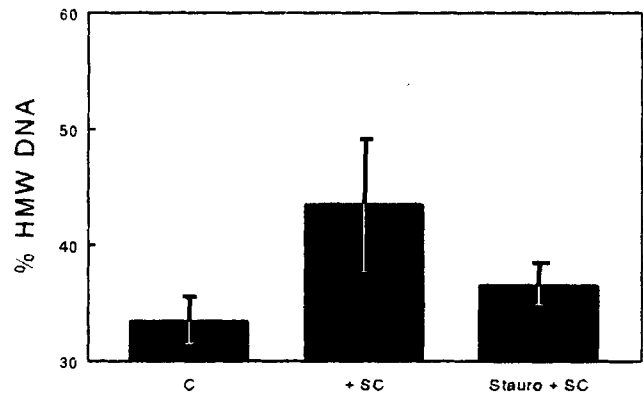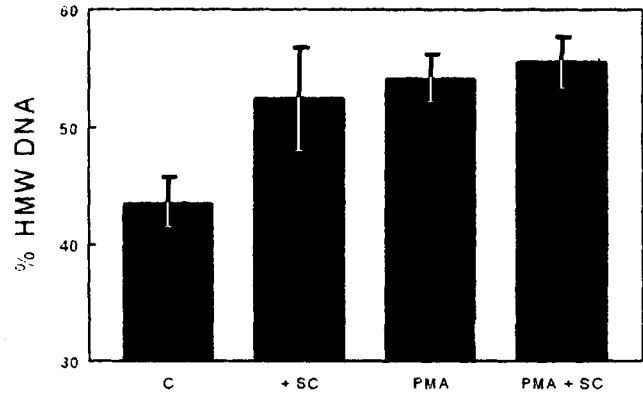

Figure 6. Role of protein kinase C in DNA degradation in mesenchymal cells. In these experiments, embryonic kidneys from each animal were divided into four samples for the experiments in Fig. 6, top, and into three samples for those of Fig. 6, bottom. Each assay point used kidneys from three animals. Each experiment was repeated three times. SC, embryonic spinal cord; $P < 0.05$ when compared to no additions, in top and bottom. 20 μM PMA; $P < 0.05$ when compared to no addition. 100 nM Staurosporine.

sis which can be distinguished by morphological and biochemical methods. Apoptosis is characterized by nuclear and cytoplasmic condensation and fragmentation. Its cardinal biochemical feature is DNA degradation by a mechanism that can be blocked by inhibitors of protein and RNA synthesis. Where tested, the DNA degradation was found to be calcium dependent. In necrosis the initial event is cell swelling and vacuolation. When nuclear breakdown occurs it is a late event and is not associated with condensation of the nucleus. Examination of Figs. 1 and 2 shows that the cells demonstrate condensation and fragmentation, findings characteristic of apoptosis. We have also demonstrated that inhibitors of RNA and protein synthesis reduced DNA degradation. If the DNA breakdown was produced by an artifact of the in vitro system causing cell death (presumably by necrosis) one would have expected that potent poisons such as actinomycin D and cycloheximide would have increased DNA degradation not reduce it. We also provided evidence that apoptosis occurs in situ during metanephric development further supporting our contention that apoptosis is a normal concomi-
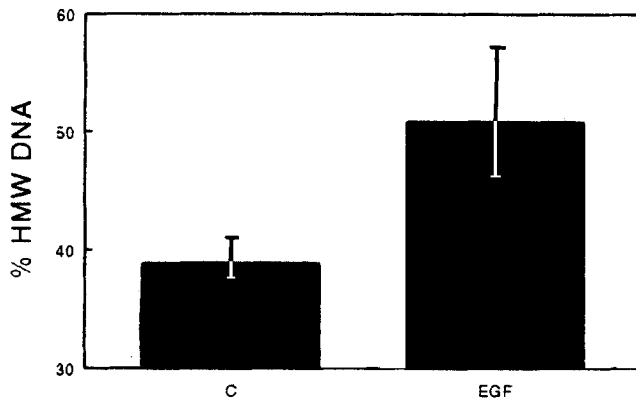
Figure 7. Effect of epidermal growth factor on DNA degradation in mesenchymal cells. Epidermal growth factor (EGF) 40 ng/ml, $n = 5$, $P < 0.05$.

tant of kidney morphogenesis. That we found that embryonic spinal cord, a heterologous inducer of metanephric development was able to prevent this process suggests that rescue from apoptosis is a necessary condition for morphogenesis. Immediately following induction, the mesenchymal cells proliferate (21). Prevention of apoptosis is obviously necessary to allow proliferation, an early step in metanephric development. Our finding that the mesenchyme is programmed for apoptosis suggests that the inductive signal must perform at least two functions, apoptosis must be first prevented following which, the mesenchyme has to convert to epithelia and differentiate.

There was a substantial quantitative difference in the amount of DNA degradation between the in vivo results of Table I and the in vitro studies of Figs. 1–7. The extent of baseline degradation in vitro was much greater. During kidney development, the ureteric bud invades the mesenchyme and a complex process occurs, in which the mesenchyme is induced to differentiate and proliferate while the ureteric bud is induced to branch. Studies on the mechanism of induction have suggested that ureteric bud cells might have to contact the mesenchymal cells in order to induce them. Hence, the process of branching during invasion allows more and more ureteric bud cells to contact mesenchymal cells. During the in vitro experiments, the inducing material, the spinal cord, was added to the outside of the mesenchymal rudiment. Hence, one likely reason for the small effect seen is that the access of the inducing cells to their target was limited to the peripheral cells. The majority of the mesenchymal cells, being at the center of the solid mass of mesenchyme, will not be induced and hence, undergo apoptosis.

Apoptosis can be induced in some tumor cells, e.g., by treatment with tumor necrosis factor (13) or by the addition of glucocorticoids to mature B cells, (26). However, it has also been observed following withdrawal of a stimulating factor such as interleukin-2 from mature T cells (3) or erythropoietin from hemopoietic cells (12). In developing dorsal root ganglia withdrawal of nerve growth factor (NGF)[1] results in programmed cell death (14). These results suggest that apoptosis might be used in two classes of processes. In one, a stimulus is needed to activate cell suicide. In the other model, the suicide genes are continuously activated but are

suppressed by factors that are produced by other cells. The second class of mechanism received recent support from the identification of the *ced-9* gene in *C. elegans.* Genetic analysis demonstrated that a gain of function mutation of *ced-9* resulted in the survival of cells of a large number of lineages (8). These studies demonstrate that apoptosis needs to be prevented in a variety of cells to assure survival. The relevance of these studies to metanephric development is highlighted by our finding that induction must rescue the metanephric cells before tubulogenesis can occur.

The factor that mediates this rescue needs to be determined. Whether it is the same as that which causes induction awaits its isolation. The metanephric mesenchyme is already known to produce or respond to several growth factors. Recently, it was found that NGF receptors are present in developing kidney mesenchyme and that antisense suppression of the NGF receptor resulted in interruption of the developmental program of the induced metanephric mesenchyme (23). Similarly, addition of antibodies to insulin like growth factor or transforming growth factor alpha prevented tubulogenesis (19, 20). However, it is not possible using these studies to determine the precise stage at which these factors act. One mechanism by which soluble growth factors might affect renal development is by rescuing metanephric mesenchyme from apoptosis. Our studies demonstrating that apoptosis can be prevented by EGF or protein kinase C activators raises the possibility that a factor which activates a tyrosine kinase (e.g., EGF) or one that activates protein kinase C might rescue mesenchyme from apoptosis. Recent studies have suggested that for NGF action to occur, the low affinity NGF receptor must bind to another receptor of the tyrosine kinase type to form a high affinity receptor (2). That we found that epidermal growth factor, another activator of tyrosine phosphorylation, prevents DNA degradation suggests that activation of tyrosine kinases might be involved in the prevention of apoptosis.

A chromosomal translocation at the *bcl2* locus results in a lymphoma suggesting that prevention of apoptosis is important for unrestricted growth (10). The *bcl2* gene is a protein located in the inner mitochondrial membrane but its function is unknown. Recent studies in germinal follicle lymphocytes have suggested that inhibition of apoptosis (by activation of the *bcl2* oncogene) is not sufficient for proliferative growth which requires the additional activation of a c-myc type of oncogene (15, 25). Hence, the inhibition of apoptosis in this model system appears to require at least two processes. Whether they are acting in parallel or in series, remains to be determined. That epidermal growth factor and phorbol esters can both prevent apoptosis but do not cause tubulogenesis suggests that the prevention of apoptosis in this system might also require the activation of at least two processes. Whether a single molecule is capable of performing both functions or two or more factors are required remains to be discovered. In addition, the same or additional factors will be needed to convert the rescued mesenchyme to epithelial cells and to initiate nephrogenesis. It is interesting in this regard that a variety of antibodies to growth factors and extracellular matrix proteins have been found to inhibit kidney development in vitro. Since kidney development occurs in many stages it would be important to develop assays that would allow definition of the stages at which these inhibitory factors work.

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1. *Abbreviation used in this paper:* NGF, nerve growth factor.

The presence of apoptosis implies that only a fraction of the mesenchyme eventually forms the mature epithelium. Since induction results in an early phase of proliferation (21), the fraction of mesenchyme that gets selected for differentiation is unknown but is likely to be small since DNA degradation appears to be fast. It has been suggested that cell to cell contact might be responsible for induction (6, 21). If this turns out to be correct, it is likely that only a limited number of targets (mesenchyme cells) would be accessible to the ureteric bud cells. This might be the explanation of the small effect of the embryonic spinal cord on DNA degradation. However, it is also possible that the inducing material is produced in limiting amounts.

Metanephric apoptosis is fast and the natural inducer (the ureteric bud) originates from a distant source which has to travel to contact the mesenchyme; hence, metanephric development has to occur within a limited time period. Because of this, it is perhaps not surprising that congenital absence of one kidney in humans is such a common occurrence, appearing in 1 in 1,000 live births.

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