Original Article

Distribution of TNFα-like proteins correlates with some regions of programmed cell death in the chick embryo

MICHAEL A. WRIDE[1], PETER H. LAPCHAK[2] and ESMOND J. SANDERS[1]*

Departments of Physiology[1] and Medicine[2], University of Alberta, Edmonton, Alberta, Canada

ABSTRACT Early chick embryos have previously been shown to express tumor necrosis factor-alpha-cross-reactive proteins (TNFα-CRPs) in a developmentally regulated manner, thus implicating these proteins in programmed cell death and in tissue remodeling. In this study, cells undergoing DNA fragmentation have been identified, using terminal deoxynucleotide transferase (TdT) mediated dUTP-biotin nick-end-labeling (TUNEL), during the embryonic development of the chick, between stages 18 and 29. DNA fragmentation is indicative of cells undergoing programmed cell death. TUNEL-positive cells were identified in several well documented areas of programmed cell death, including the limb buds, the heart, spinal motoneurons, dorsal root ganglia, and the ventral horn of the neural tube. In addition, other areas of cell death were identified including the floor plate and the mesonephros. In several locations, a close correlation was noted between the presence of TUNEL-positive cells and regions of TNFα-immunoreactivity. These regions included the ventral horn and marginal zone of the neural tube, spinal motoneurons, paravertebral ganglia, parts of the myotome, mesenchyme of the body wall, and the mesonephros. In addition, using the TNFα-sensitive L929-8 bioassay it was shown that homogenate of stage 18 chick embryos is cytotoxic to L929-8 cells and that this toxicity can be reduced using neutralizing antibodies to mouse TNFα. This bioassay allowed us to estimate the mean concentration of TNFα-like activity in embryo homogenate, which is within the range of physiological (pg/ml) levels of TNFα found in other systems. These results suggest that proteins with TNFα-like activity may have a role in programmed cell death in some tissues during early chick embryo development.

KEY WORDS: *chick embryo, development, TNFα, apoptosis*

Introduction

It is well established that normal embryonic development is accompanied by a coordinated pattern of cell deletion (Snow, 1987; Ellis *et al.*, 1991). This "programmed cell death" is seen very early in development (Bellairs, 1961), and has a vital role in the differentiation of many tissues and organs, including: the limb buds (Hinchliffe, 1981), the nervous system (Oppenheim, 1991; Barinaga, 1993) and the heart (Pexieder, 1975). Programmed cell death is frequently characterized morphologically by apoptosis, which is identifiable by early nuclear compaction, cytoplasmic condensation and blebbing of both nuclear and plasma membranes (reviewed by Kerr *et al.*, 1987).

Previously, programmed cell death could only be detected *in situ* using vital dyes taken up through the perforated membranes of dying cells (Saunders *et al.*, 1962; Jeffs and Osmond, 1992); however, loss of membrane integrity is a relatively late event in cell death (Vaux, 1993). Alternatively, cells undergoing apoptosis can be identified using the purely morphological criteria described above (Kerr *et al.*, 1987), but dying cells are removed very rapidly during programmed cell death and are not easily identifiable in tissue sections.

A technique that allows identification of cells undergoing apoptosis *in situ* is that of TUNEL (Gavrieli *et al.*, 1992). This method is based on the fact that apoptosis is usually accompanied by fragmentation of nuclear DNA between nucleosomes by endogenous endonucleases (Gerschenson and Rotello, 1992). Although several recent studies have revealed that the morphology associated with apoptosis can occur without DNA fragmentation in some instances (Cohen *et al.*, 1992; Tomei *et al.*, 1993; Zakeri *et al*, 1993), this fragmentation remains an essential feature of programmed cell death in many cases, and may be an early event in

Abbreviations used in this paper: TUNEL, terminal deoxynucleotide transferase mediated dUTP-biotin nick-end labeling; TdT, terminal deoxynucleotide transferase; TNFα, tumor necrosis factor-alpha; TNFα-CRPs, tumor necrosis factor-alpha-cross-reactive proteins; PBS, phosphate buffered saline; TBS, tris-buffered saline; DDW, distilled de-ionised water; SSC, saline sodium citrate; SM, skimmed milk; AEC, 3-amino-9-ethyl carbazole; DMF, di-methyl formamide; $p75^{NGFR}$, p75 low affinity nerve growth factor receptor.

***Address for reprints:** Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. FAX: 403-492-8915.

0214-6282/94/$03.00

Printed in Spain

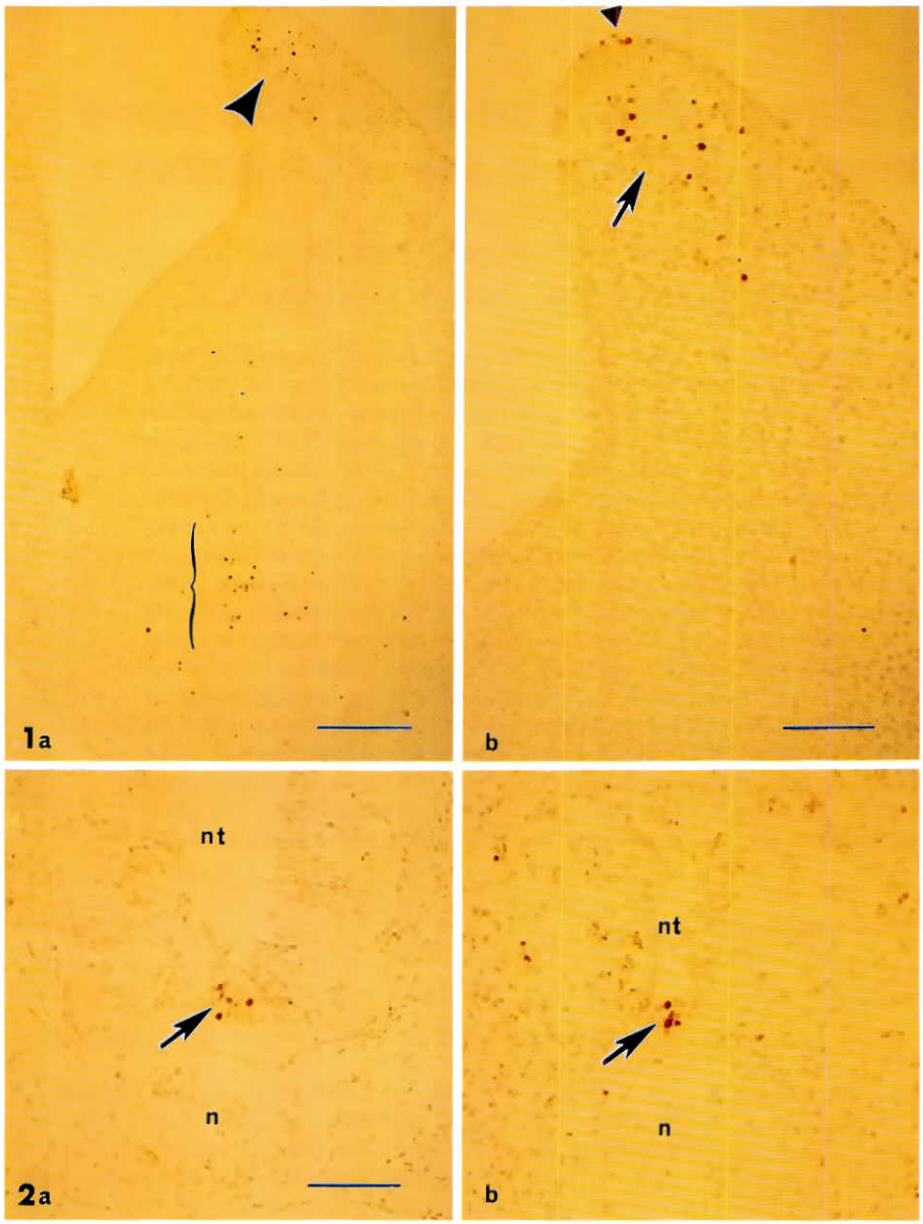

Fig. 1. Section through the wing bud (day 4, stage 23). (a) *TUNEL-positive cells are present in the central core cells (bracketed) and in the distal subridge mesenchyme (arrowhead). Bar, 100 μm.* **(b)** *Higher magnification of* **(a)** *showing details of the TUNEL-positive cells in the apical ectodermal ridge (arrowhead) and distal subridge mesenchyme (arrow). Bar, 50 μm.*

Fig. 2. Sections through the nerve cord. (a) *Day 3 (stage 18). Section through the neural tube (nt) and notochord (n) at the level of the wing buds. TUNEL-positive cells are present in the floor plate of the neural tube (arrow). Bar, 50 μm (a,b).* **(b)** *Day 6 (stage 29). Section through the notochord and neural tube in the tail bud where TUNEL-positive cells persist in the floor plate (arrow).*

the cell death pathway. TUNEL allows detection of cells undergoing DNA fragmentation, by incorporation of labeled nucleotides at free 3'-OH ends of nicked DNA, using TdT. These transient dying cells can therefore be detected cytochemically in tissue sections. Furthermore, if cells are both TUNEL-positive and display characteristic morphological features, they can then be designated as truly apoptotic.

It has been suggested that homeobox genes may have a role in cell death during chick limb development (Coelho *et al.*, 1993) and the expression of the homeobox gene *msx* correlates with apoptosis in the neural crest of the chick hindbrain (Graham *et al.*, 1993). Proto-oncogenes may also be involved, as suggested by the recent report of *c-fos* expression preceding programmed cell death *in vivo* (Smeyne *et al.*, 1993). In the central core of mouse embryo limb buds, apoptosis may be accompanied by a transient increase in tissue transglutaminase expression (Jiang and Kochhar, 1992), while the testosterone-repressed prostate message-2 gene accompanies programmed cell death of interdigital tissue (Buttyan *et al.*, 1989). The product of the polyubiquitin gene has also been shown to accompany programmed cell death in some cases (Schwartz, 1991). However, the identity of many of the proteins expressed during programmed cell death *in vivo* remains undetermined. This is particularly true of the putative "suicide" or "killer" proteins that are proposed to actually initiate the events of programmed cell death.

In a previous study (Wride and Sanders, 1993a), the existence of a developmentally regulated expression of TNFα-CRPs has been reported during the early development of the chick embryo. Considering the ability of TNFα to instigate DNA fragmentation and apoptosis in sensitive cells (Larrick and

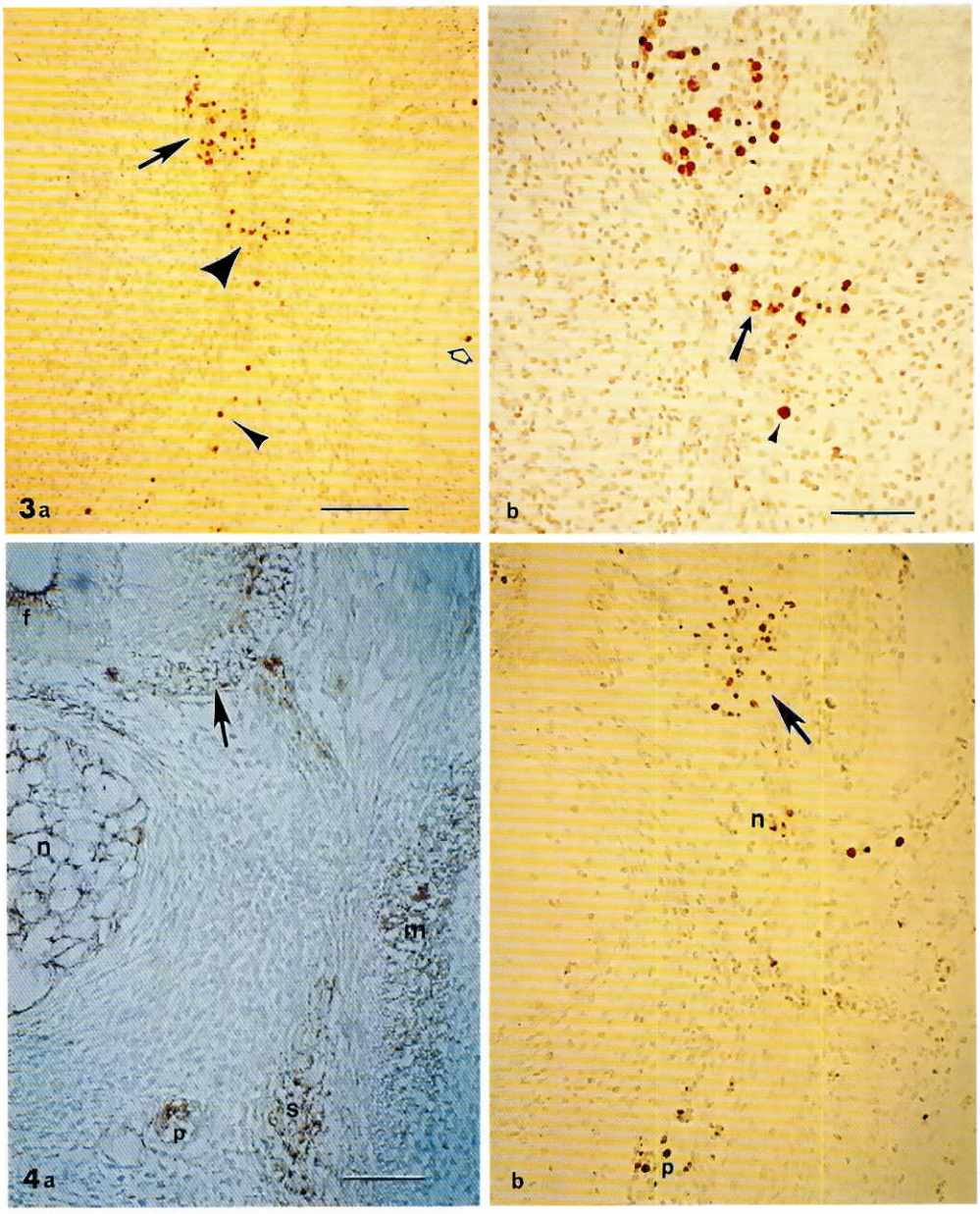

Fig. 3. Section through the trunk at the level of the wing bud (day 6, stage 29). (a) *Massive cell death in the ventral aspect of the dorsal root ganglion (arrow) the ventral nerve (large arrowhead) and the spinal nerve (small arrowhead). Note the positive nucleus in the notochord (open arrow). Bar, 100 μm.* **(b)** *Higher magnification of* **(a)**. *Some TUNEL-positive cells in nervous tissue appear to exhibit the characteristic blebbing morphology of apoptosis (arrow) while others appear to look homogeneous (arrowhead). Bar, 50 μm.*

Fig. 4. Closely adjacent sections through the trunk (day 5, stage 26). (a) *Immunocytochemical localization of TNFα. Staining is present in the notochord (n), in the marginal zone of the neural tube (arrow), in the myotome (m), in the paravertebral ganglia (p), in the floor plate (f) and in the spinal nerve (s). Bar, 50 μm (a,b).* **(b)** *Section through the trunk in the same region of the same embryo as in* (a), *but stained using TUNEL. TUNEL-positive cells are present in the ventral horn and marginal zone of the neural tube (arrow), in the ventral nerve root (n) and in the paravertebral ganglion (p). In other sections, labeled nuclei could also be found in the floor plate, notochord, and myotome.*

Wright, 1990; Wright *et al.*, 1992) and the developmental distribution of TNFα-CRPs, it was speculated that one of the roles of proteins with TNFα-like activity in embryos could be the initiation of programmed cell death at specific sites and times during development.

In the present report, TUNEL was used to detect cells undergoing programmed cell death and, in some locations, a close correlation was noted between the expression of TNFα-CRPs and cells that are undergoing DNA fragmentation detected by TUNEL. These tissues included: the ventral horn and marginal zone of the neural tube, spinal motoneurons, paravertebral ganglia, parts of the myotome, mesenchyme of the body wall, and the mesonephros. We also show that chick embryo homogenate possesses endogenous TNFα-like cytotoxic activity, which could be reduced when homogenate was incubated with neutralizing antibodies to TNFα. Thus, TNFα-CRPs could be involved in programmed cell death in some tissues during early chick embryo development.

Some of this work has been published previously in abstract form (Wride and Sanders, 1993b).

Results

Using TUNEL, we have been able to identify cells undergoing DNA fragmentation during the early development of the chick embryo. These cells are present in several well characterized regions of cell death including the limb buds, the heart, spinal motoneurons, dorsal root ganglia, and the ventral horn and marginal zone of the neural tube, confirming that TUNEL is a reliable method for the detection of cells undergoing programmed cell

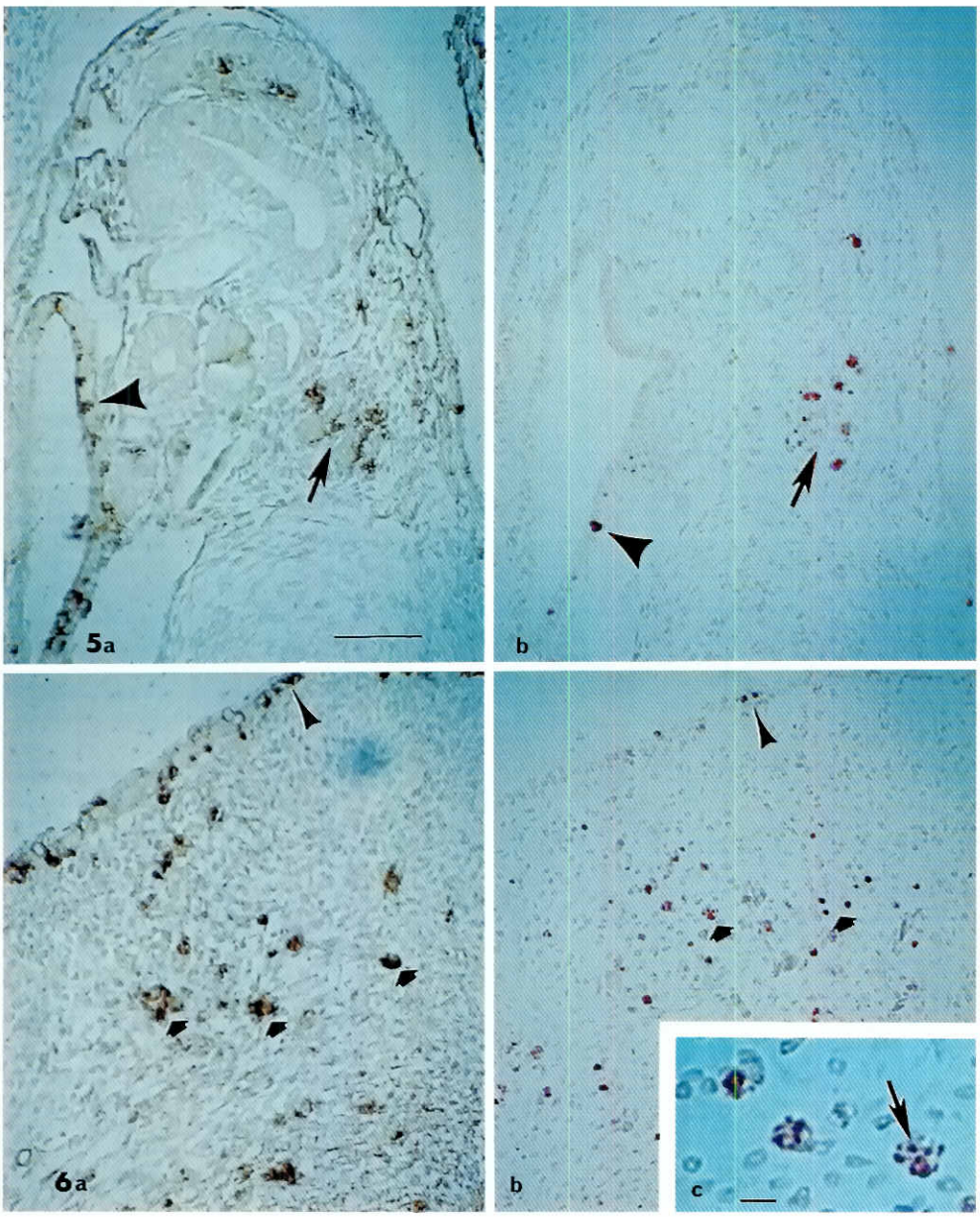

Fig. 5. Closely adjacent sections through the mesonephros (day 5, stage 26). (a) *Immunocytochemical localization of TNFα in the mesonephric mesenchyme (arrow) and in the wall of the Wolffian duct (arrowhead). Bar, 50 μm (Figs. 5a,b and 6a,b).* **(b)** *Section through the mesonephros in the same region of the same embryo as in* **(a)**, *but stained using TUNEL. Dying cells identified by TUNEL are present in both the mesonephric mesenchyme (arrow) and in the wall of the Wolffian duct (arrowhead).*

Fig. 6. Closely adjacent sections through the body wall adjacent to the gut (day 6, stage 29). (a) *Immunocytochemical localization of TNFα in the mesenchyme and in particular in several intensely stained foci (arrows). Staining is also present in the ectoderm (arrowhead).* **(b)** *Section through the body wall in the same region of the same embryo as in* **(a)**, *but stained using TUNEL. TUNEL-positive cells are present in the mesenchyme (arrows) and ectoderm (arrowhead) of the body wall.* **(c)** *Higher magnification of* **(b)** *showing apoptotic cells in the mesenchyme. Cells show the membrane blebbing characteristic of apoptosis (arrow). Bar, 10 μm.*

death. In addition, other areas of cell death were identified, including the floor plate, some cells of the myotome, and the mesonephric mesenchyme and Wolffian duct.

The limb buds (stage 23)

Cells undergoing DNA fragmentation were detected in the distal subridge mesenchyme and apical ectodermal ridge (Fig. 1a,b) and in the central core of the limb bud (Fig. 1a).

The floor plate (stages 18 and 29)

A region of cell death was identified in the floor plate of the neural tube. DNA fragmentation, identified by TUNEL, was present in several cells in this region of the neural tube. Dying cells in the floor plate were first identified at the level of the wing buds at stage 18 (Fig. 2a). This pattern of cell death was still prevalent in the cells of the floor plate in the tail bud of embryos at stage 29 (Fig. 2b).

The nervous system (stage 29)

Massive cell death in nervous tissue was revealed using TUNEL. As can be seen in Fig. 3a and 3b, TUNEL-positive cells are present in the ventral aspect of the dorsal root ganglion and in the spinal motoneurons. Under higher magnification (Fig. 3b), some of the nuclei exhibit the morphology that has been associated with that of apoptosis (condensed chromatin and membrane blebbing). Other nuclei look dense and homogeneous, which is the more commonly described appearance of dying neurons.

In several regions, a positive correlation was found between the presence of TUNEL-positive cells and regions of TNFα-immunoreactivity. These regions included the ventral horn of the neural tube, spinal neurons, paravertebral ganglia, mesenchyme of the body wall, the mesonephric mesenchyme and the Wolffian duct, some parts of the myotome, and cranial nerves and ganglia.

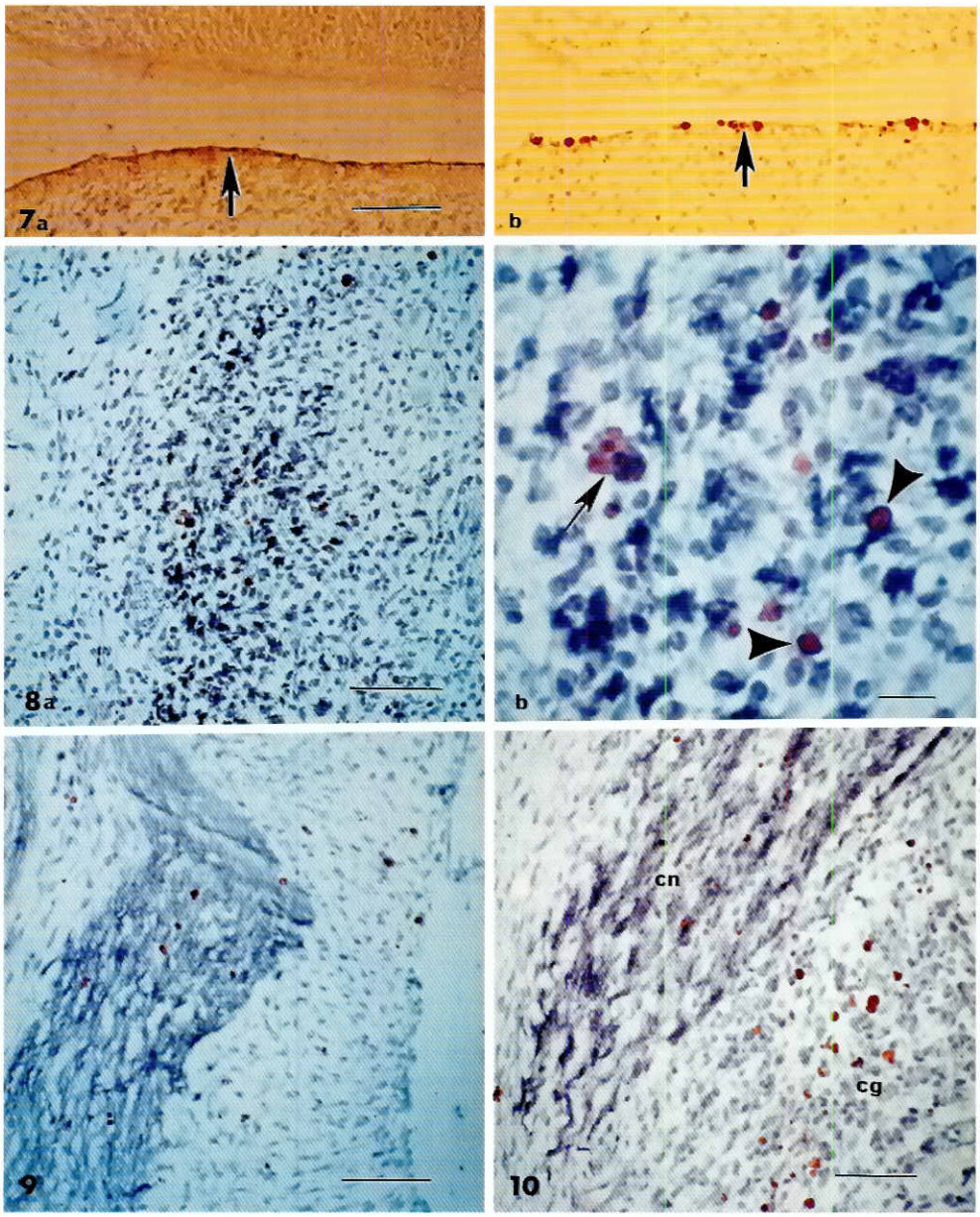

Fig. 7. Section through the body wall adjacent to the limb bud (day 6, stage 29). (a) *Immunocytochemical localization of TNFα in the ectoderm of the body wall (arrow). Bar, 50 μm (a,b).* **(b)** *Adjacent section through the body wall in the same region of the same embryo as in* **(a)** *showing TUNEL-positive cells in the ectoderm (arrow).*

Fig. 8. Double labeled section through the trunk (day 6, stage 29). (a) *TNFα-immunoreactivity in the myotome (dark blue) and apoptotic cells (red). Bar, 50 μm.* **(b)** *Higher magnification of* **(a)**. *One cell (arrow) showing the classic membrane blebbing of apoptosis, is TUNEL-positive (red), and also appears to be TNFα-immunoreactive (dark blue). Other TUNEL-positive cells appear to be stained dark blue for TNFα at their peripheries (arrowheads). Bar, 10 μm.*

Fig. 9. Double labeled section through the heart (day 6, stage 29). *TNFα immunoreactivity is seen primarily in the developing cardiac muscle of the ventricle wall (dark blue) while TUNEL-positive cells (red) are also seen in this tissue. Bar, 50 μm.*

Fig. 10. Double labeled section through the head (day 5, stage 26). *The cranial nerve (cn) is immunoreactive for TNFα (dark blue) and contains TUNEL-positive cells (red), while the adjacent cranial ganglion (cg) also contains many TUNEL-positive cells (red). Bar, 50 μm.*

The trunk (stage 26)

Immunocytochemical localization of TNFα-immunoreactivity using monoclonal anti-mouse TNFα (Fig. 4a) revealed an identical pattern of expression to that described previously using a polyclonal antibody (Wride and Sanders, 1993a). When TUNEL was performed on sections adjacent to those stained with the antibodies to TNFα, it became apparent that a positional correlation existed in some tissues between the expression of TNFα-CRPs and the presence of fragmented DNA in cells of the same tissues. Dying cells were present in the ventral horn of the neural tube and in the marginal zone (Fig. 4b). The marginal zone was an area of intense TNFα immunoreactivity at this stage (Fig. 4a), while there was less immunoreactivity in the ventral horns. The ventral nerve was intensely immunoreactive and TUNEL-positive cells were observed in this tissue (compare Fig. 4a and 4b). The paravertebral ganglia were also highly TNFα-immunoreactive at this stage and a direct correlation was evident between the presence of TNFα-immunoreactivity in this tissue and the presence of dying cells (compare Fig. 4a and 4b).

The mesonephros (stage 26)

Cells in the mesonephric mesenchyme and in the walls of the Wolffian duct were found to be undergoing cell death at this stage of development. TNFα-immunoreactivity was also present in these tissues (Fig. 5a and 5b).

Mesenchyme of the body wall (stage 29)

It was noted that a particular area of mesenchyme adjacent to the gut had a high number of TUNEL-positive cells, many of which exhibited the classical apoptotic features (Fig. 6b and 6c). In addition, some cells of the ectoderm appeared to be dying (Fig. 6b). In an adjacent section, TNFα- immunoreactivity was present

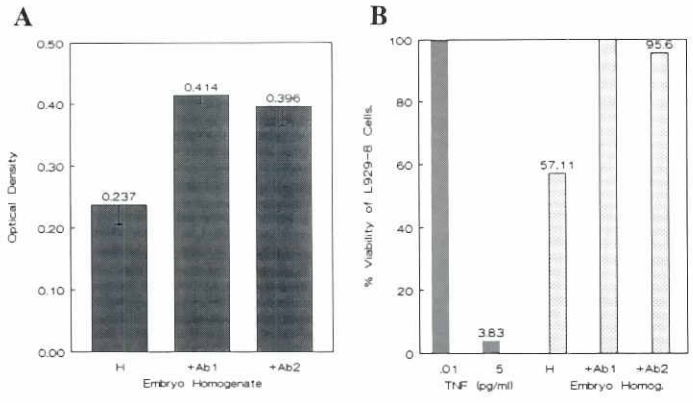

Fig. 11. The L929-8 TNFα bioassay. (a) *The effect of day 3 (stage 18) chick embryo homogenate at a final dilution of 1:36 on the optical density of L929-8 cells alone (H) or in the presence of neutralizing antibodies to TNFα (Ab1, Genzyme and Ab2, Genentech). The optical density is significantly reduced (implying increased cell death) in the presence of embryo homogenate compared to that with embryo homogenate in the presence of TNFα-specific neutralizing antibodies (p<0.005, Ab1 and p<0.005, Ab2).* **(b)** *The percent viability of L929-8 cells challenged with exogenous TNFα at concentrations of 0.01 pg/ml and 5 pg/ml or with embryo homogenate at a final dilution of 1:36 alone (H) or embryo homogenate in the presence of neutralizing antibodies to TNFα (Ab1, Genzyme and Ab2, Genentech).*

throughout the mesenchyme, but showed foci of particularly intense staining (Fig. 6a). Cells of the ectoderm were also stained with these antibodies to TNFα (Fig. 6a).

Ectoderm

TNFα-positive cells of the ectoderm of the body wall immediately adjacent to the limb bud are shown in Fig. 7a. These cells are intensely TUNEL-positive and their presence correlates with the presence of TNFα-immunoreactivity throughout the ectoderm.

The myotome

The myotome stained intensely with monoclonal antibodies to TNFα. When double labeling was used to stain cells of the myotome with antibodies to TNFα (dark blue) and TUNEL (red), it was noticed that cells in the ventral aspect of the myotome were TUNEL-positive and that this correlated with the presence of TNFα-immunoreactivity in this tissue (Fig. 8a and 8b).

The heart

Dying cells were observed in the walls of the heart using TUNEL. However, TNFα immunoreactivity in the heart did not appear to correlate wholly with the presence of these cells, although there did appear to be more TUNEL-positive cells (red) in regions of TNFα immunoreactivity (dark blue; Fig. 9).

Cranial nerves and ganglia

When double labeling was carried out on sections through the head (Fig. 10), it was observed that cranial nerves and ganglia were immunoreactive for TNFα (dark blue). These tissues were also found to contain many TUNEL-positive cells (red), but TUNEL-positive cells were also particularly abundant in immediately adjacent tissue (Fig. 10).

The L929-8 TNF bioassay

Chick embryo homogenate was incubated with TNFα-sensitive L929-8 cells, both alone and in the presence of two different neutralizing antibodies to TNFα (Ab1 and Ab2; see Materials and Methods). The optical density of neutral red staining of the cells was used as a measure of the level of L929-8 cell death that had occurred (the lower the optical density the greater the cell killing; Fig. 11a). With embryo homogenate, the mean optical density was 0.237 (±0.025 SE, n=6), compared with mean values, obtained using embryo homogenate with antibodies to TNFα, of 0.414 (±0.008 SE, n=4; Ab1) and 0.396 (±0.033 SE, n=4; Ab2). The value obtained with embryo homogenate alone was significantly different from the value obtained with embryo homogenate with either neutralizing antibody to TNFα ($p<0.005$, Ab1 and $p<0.005$ Ab2).

The percent viability of L929-8 cells was calculated from the optical density values presented above and from values obtained in the presence of exogenous TNFα (Fig. 11b). Maximum percent viabilities were observed with 0.01 pg/ml TNFα (100%) and with embryo homogenate with neutralizing antibodies to TNFα (100% Ab1 and 95.6% Ab2). The minimum percent viability under the present conditions was seen with 5 pg/ml TNFα (3.83%) while embryo homogenate alone gave a value of 57.11%. It was found that the presence of embryo homogenate had the effect of increasing apparent cell viability by several percent in all assays, presumably by promoting L929-8 cell proliferation, therefore the data presented above were normalized to 100% for cells in the presence of both antibody and embryo homogenate to take into account this proliferative effect.

Using a standard curve of optical density against increasing concentrations of exogenous TNFα (0.01 pg/ml to 5 pg/ml) added to L929-8 cells, it was possible to calculate the mean concentration of bioactive TNFα present in a 1:36 dilution of stage 18 embryo homogenate. From this value the concentration of bio-active TNFα in whole stage 18 embryos could be calculated. This was found to be 10.3 (±0.5 SE, n=6) pg/ml of undiluted embryo homogenate without antibodies, compared to complete elimination of TNFα-like activity in the presence of neutralizing antibodies.

Discussion

Programmed cell death is a term that is familiar to workers in the fields of both developmental biology and immunology. However, workers in each of the two fields view programmed cell death from different perspectives (Schwartz and Osborne, 1993). Developmental biologists understand the phenomenon as the deletion of cells in a spatially and temporally restricted manner during normal embryonic development as a result of a physiological stimulus, while immunologists restrict their definition to the death of any cell that requires *de novo* protein expression to bring about its own demise, regardless of the nature of the stimulus (physiological or non-physiological). It has been noted previously (Wride and Sanders, 1993a) that proteins which are cross-reactive with antibodies to TNFα are expressed in some tissues which are known to undergo programmed cell death during early chick embryo development. These observations have now been extended using TUNEL to detect cells undergoing the DNA fragmentation indicative of programmed cell death. The present study has shown that a positive correlation exists between TNFα-immunoreactivity and programmed cell death in some locations in the embryo and that TNFα-like cytotoxic activity is present in chick embryo homogenate. These results might suggest that proteins with TNFα-like activity could represent some of the putative "killer proteins" synthesized in tissues undergoing developmental programmed cell death, just

as they are in immunological programmed cell death (Hernández-Caselles and Stutman, 1993; discussed below).

The role of TNFα in pathology and inflammation is well documented (Vassalli, 1992), but its physiological functions are not as well understood. Hernández-Caselles and Stutman (1993) have noted that the intrathymic stage of T-cell development is characterized by high rates of cell proliferation and cell death. These authors reasoned that since TNFα is capable of instigating both the death and proliferation of cells, it could have a role in intrathymic T-cell development. This argument is analogous to our proposal that since normal embryonic development is similarly characterized by high rates of cell proliferation and death, proteins with TNFα-like activity, when produced locally and at physiological concentrations, could have a role in embryogenesis (Wride and Sanders, 1993a, 1994). Indeed, Hernández-Caselles and Stutman (1993) showed that TNFα influences thymocyte proliferation and apoptosis during intrathymic T-cell development.

It has been suggested that the TUNEL method, and related *in situ* nick-end-labeling techniques, may be *selective* rather than *specific* for cells undergoing programmed cell death; i.e. necrotic cells are also labeled (Ansari *et al.*, 1993). However, as these authors point out, necrotic cells are easily identifiable in tissue sections as large areas of labeled cells rather than scattered TUNEL-positive nuclei such as those that we observed in our experiments. Indeed, necrotic areas with many TUNEL-positive nuclei were visible in some of our sections, where tissue had obviously been damaged during fixation or processing (not shown). Also, when combined with consideration of the classic morphological criteria described for apoptotic cells, it was apparent that many TUNEL-positive cells also showed this morphology (see Fig. 6c). Other cells, though not showing an apoptotic morphology, were TUNEL-positive and were isolated from each other, supporting the contention that TUNEL is able to detect DNA fragmentation in dying cells at early stages, before the morphological criteria for apoptosis are exhibited. Finally, it has been considered that cell division and differentiation is also accompanied by the introduction of nicks into DNA (Ansari *et al.*, 1993). However, we were able to optimize the TUNEL technique so that a very low level of background staining was observed, which was significantly less than the staining observed in TUNEL-positive nuclei, suggesting that any DNA nicks in normal cells were below the level of detection. Therefore, we consider that the TUNEL method proved to be very effective in detecting cells undergoing programmed cell death *in situ*.

The nervous system

Using TUNEL, we have confirmed the finding that programmed cell death accompanies the normal development of the chick nervous system. In the lateral motoneurons of the lumbar spinal cord of the chick embryo, it has been noted that 40% of the neuron population degenerates between day 5.5 and day 9 of incubation (Chu-Wang and Oppenheim, 1978). We have identified dying cells in the lateral motor columns of the chick at day 5 (stage 26) and day 6 (stage 29) and we have also shown that this cell death is accompanied by DNA fragmentation. In addition, dying cells are identified in the paravertebral ganglia and in the spinal motoneurons at this stage. It is confirmed here that TNFα-immunoreactivity can be detected in these areas of the nervous system during these stages of development. This lends support to our previous proposal (Wride and Sanders, 1993a) that TNFα-CRPs may be involved in programmed cell death in the nervous system. It is possible that the TUNEL-positive cells in the region of the spinal motoneurons are actually Schwann cells that are degenerating following the loss of axons due to programmed motoneuron cell death at this stage, since it has been suggested that, in the rat at least, the number of Schwann cells in a developing nerve is matched by the number of axons that are present (Harris and McCaig, 1984). Alternatively, these TUNEL-positive cells could represent the avian equivalent of Schwann cell precursors, which are intermediates in the development of Schwann cells from neural crest cells and have been shown to undergo programmed cell death in the rat embryo (Jessen *et al.*, 1994). Thus, TNFα-CRPs could be one family of "killer proteins" present within the developing nervous system. Dying cells have also been identified in the dorsal root ganglia, a tissue which has been consistently negative in our studies of TNFα immunoreactivity. This suggests that if TNFα-like proteins are involved in programmed cell death in the nervous system, they may be involved only in certain regions. Furthermore, the identification of cells undergoing DNA fragmentation in the floor plate is a novel finding and suggests that cell death may be involved in the differentiation of this important region of the nervous system. Very recently, Homma *et al.* (1994) have also reported cell death in the floor plate at this time. In the present study, some dying cells in the nervous system showed classic apoptotic morphological features while others had nuclei which stained homogeneously. The latter could represent nuclei in the early stages of DNA fragmentation, which have yet to undergo the morphological changes associated with apoptosis.

The mesonephros

The presence of TUNEL-positive cells in two different areas of the mesonephros, the mesonephric mesenchyme and the Wolffian duct, was particularly interesting. The development of the mesonephros has been described by Friebová (1975). The observation that programmed cell death is present in the mesonephros is new and it complements recent reports of programmed cell death accompanying the development of the metanephros (Koseki *et al.*, 1992; Coles *et al.*, 1993). It has been proposed that the metanephric mesenchyme is programmed for apoptosis and that there are two steps to the inductive signal from the ureteric bud to the mesenchyme. The first is the prevention of apoptosis in the mesenchyme while the second is the conversion of the mesenchyme to epithelium, followed by differentiation (Koseki *et al.*, 1992). Similar morphogenetic events may occur in the mesonephros during early chick development and since dying cells have been identified in the mesonephric mesenchyme, this tissue may also have an inherent potential to undergo programmed cell death. The chick mesonephric mesenchyme and the Wolffian duct have also been shown here to have TNFα-immunoreactivity, indicative perhaps of a role for TNFα-CRPs in programmed cell death in these tissues. The significance of dying cells in the mesonephros at these stages remains undetermined, but it is unlikely to be the cause of regression of this tissue, since this is not thought to occur until the 9th embryonic day (Friebová 1975).

The limb buds and the heart

The developing limbs have proven to be an excellent model system in which to study the apparent paradox of cell death during development (reviewed by Hinchliffe, 1981). TUNEL-positive cells were identified in several regions including the opaque zone, within the central core cells, and in both the apical ectodermal ridge and the distal subridge mesenchyme. The presence of programmed cell death in the distal subridge mesenchyme of the chick is a new

observation, since previously cell death in this region has only been detected following the removal of the apical ectodermal ridge (Rowe *et al.*, 1982). The apical ectodermal ridge is clearly intact in our studies (see Fig. 1b), suggesting that the distal subridge mesenchyme is a region of naturally occurring programmed cell death. The presence of TNFα immunoreactivity in the developing non-chondrifying mesenchyme of the limbs has previously been reported (Wride and Sanders, 1993a), but in the present study a complete correlation between the expression of TNFα-CRPs and dying cells identified by TUNEL, in all regions of the limb bud, was not made.

The heart is another well known site of programmed cell death (Pexieder, 1975). Cell death was identified here in the heart throughout the stages that were examined, but dying cells were particularly evident by stages 26-29. Previously, the presence of TNFα-CRPs in heart tissue was shown in Western blots of stage 23 embryos (Wride and Sanders, 1993a). TNFα immunoreactivity is shown here to be present in the heart, particularly in the trabeculae and in the developing muscle of the walls of the ventricles. However, the expression of TNFα-CRPs in the heart, like the limb, was not restricted to regions where cell death occurred. This does not necessarily preclude a role for TNFα-CRPs during programmed cell death in the heart or limb, but rather suggests that they may have additional roles in these tissues, perhaps in extracellular matrix remodeling and as growth and proliferation factors (Wride and Sanders, 1994).

The L929-8 TNFα bioassay

The L929-8 cell bioassay was used to detect TNFα-like activity in sterile homogenate from stage 18 embryos. The sensitivity of this cell line is highly specific for TNFα and TNFß (for a complete discussion see Branch *et al.*, 1991). The embryo homogenate in the absence of antibodies to TNFα significantly reduced the viability of the L929-8 cells compared to homogenate in the presence of antibodies to TNFα, implying that the death of L929-8 cells is specifically due to TNFα-like cytotoxic factors present within the embryo homogenate. This result supports the contention that the TNFα-like activity in the embryo homogenate is specifically due to the TNFα-CRPs). However, at the present time, the exact nature of the TNFα-CRPs remains uncertain, since they have yet to be cloned and sequenced (for discussion, see Wride and Sanders, 1994). Furthermore, the mean concentration of TNFα-like activity in embryo homogenate was found to be 10.3 pg/ml, a value which is within the range of physiological (unstimulated), rather than pathophysiological, levels of TNFα. It is important to emphasize that this is a *mean* value and that the two TNF receptors have Kds in the pg/ml range (Rothe *et al.*, 1992). Therefore, in order to have biological effect and assuming that they can interact with the TNF receptors (since they do so, at least with the TNF receptor that mediates cytotoxicity, in the L929-8 bioassay), TNFα-CRPs must be present in concentrations that exceed the Kds for their receptors in embryonic tissues. From our immunohistochemical data (Wride and Sanders, 1993a; Wride and Sanders, 1994; the present results), it is clear that the distribution of TNFα-CRPs in the embryo is not homogeneous, suggesting that these proteins are present in sufficiently high concentrations to bind to TNF receptors in tissues in which they are expressed. The results of the bioassay described here support data obtained by Yamasu *et al.* (1989) who showed that mouse fetuses secrete biologically active TNFα in the absence of exogenous stimuli, such as treatment with lipopolysaccharide.

Concluding comments

Programmed cell death is followed by ingestion of dying cells by macrophages or other phagocytes (Savill *et al.*, 1993). Macrophages are potent producers of TNFα (Vassalli, 1992), therefore it is possible that at least some of the TNFα immunoreactivity, which we have detected in the developing chick, could be produced by macrophages moving into areas of cell death in order to clear dead cells. However, phagocytes are unlikely to be the only producers of TNFα in developing embryos, since Yamasu *et al.* (1989) have shown that macrophages are not the primary producers of TNFα during mouse development. Therefore, production of TNFα-like proteins may potentially be a cause as well as a consequence of cell death during development.

It may be significant, when suggesting a potential role for TNFα-like proteins in programmed cell death, that the p75 low affinity nerve growth factor receptor ($p75^{NGFR}$) constitutively induces neural cell death unless it is bound by NGF (Rabizadeh *et al.*, 1993). This is particularly interesting since $p75^{NGFR}$ is a member of an emerging family of receptors and ligands belonging to the NGF/TNF receptor family (reviewed by Bazan, 1993). In the nervous system, loss of support by trophic factors, such as NGF, could result in the death of neurons due to apoptosis initiated by $p75^{NGFR}$ or perhaps due to the direct action of TNFα-like proteins on their receptors, as is suggested by the present results. Thus, TNFα-like proteins and their receptors, NGF, trophic factors, and proto-oncogenes such as *bcl-2* could be part of a general cell death system (the "social controls"; Raff *et al.*, 1993), which is used to initiate and control cell death during embryonic development. In fact, an involvement for TNFα in the "social controls" of cell survival and cell death has already been suggested for its roles in T-cell production in the thymus (Hernández-Caselles and Stutman, 1993).

In conclusion, this study has shown that chick embryos possess endogenous TNFα-like cytolytic activity at early stages of development, these TNFα-like proteins are produced at physiologically relevant concentrations in the embryo, and they are expressed in some tissues undergoing programmed cell death detected by TUNEL. This lends support to the previous proposal that one potential role for TNFα-CRPs during early embryonic development could be in programmed cell death (Wride and Sanders, 1993a). Functional studies, to investigate this hypothesis, are underway. However, it is likely that there are several, if not many, mechanisms of programmed cell death in embryos and it is therefore unlikely that TNFα-like proteins are involved in *all* cases of programmed cell death. This is evident, in the present report, since some regions of cell death detected by TUNEL do not express TNFα-CRPs; e.g. the dorsal root ganglia. Indeed, it is possible that TNFα-like proteins also have important roles during development in the remodelling of the extracellular matrix, the expression of cell adhesion molecules and integrins, and in cell growth and differentiation, as well as in programmed cell death (Wride and Sanders, 1994).

Materials and Methods

Preparation of embryos for immunocytochemistry and TUNEL

White Leghorn hens' eggs were incubated at 37°C for 3 to 6 days (Stages 18-29; Hamburger and Hamilton, 1951). The embryos were removed from their yolk and rinsed and handled in Tyrode's saline. Embryos were fixed with 4% paraformaldehyde overnight at 4°C, washed in PBS (pH 7.4), dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Blocks were sectioned at 5 μm thickness.

Immunocytochemistry

Immunocytochemistry for TNFα was carried out as described previously (Wride and Sanders, 1993a) using the alkaline phosphatase-based AS/AP® kit (BioCan Scientific Inc.), and two rat monoclonal antibodies to mouse TNFα. Clone MP6-XT22 was obtained from either Endogen Inc. or abV ImmuneResponse Inc., while clone MP8-XT22 was obtained from UBI Inc. These antibodies were used at a dilution of 1:20, and were detected with the AS/AP® system in which the second antibody was replaced by biotinylated rabbit anti-rat IgG (Dimension Laboratories Inc.) diluted 1:50 in TBS (pH 7.6) plus blocking agent (supplied in the kit). Antibody preabsorbed with mouse TNFα was used as a control, as described previously (Wride and Sanders, 1993a).

TUNEL

Incorporation of biotinylated nucleotides at free 3'-OH ends of DNA in tissue sections was carried out as described by Gavrieli *et al.* (1992) with modifications according to Wijsman *et al.* (1993). Tissue sections were cleared in Hemo-De (2x5mins), rehydrated through a graded series of alcohol, and were finally immersed in DDW. Sections were then incubated for 20 min in 2xSSC buffer (0.3 M sodium chloride, 30 mM sodium citrate, pH 7) at 60°C, washed in DDW, and immersed in proteinase K buffer (10 mM Tris-HCl, pH 8) for 10 min at room temperature. This was followed by incubation with 20 μg/ml of freshly prepared proteinase K (Sigma), for 15 min at room temperature, and a final wash in DDW. Endogenous peroxidase was inactivated by covering the sections with 2% H_2O_2 in DDW, with 0.5% Tween 20 for 10 min at room temperature. Sections were then incubated in 10xTdT buffer, pH 7.2 (30 mM Trizma base, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 5 min at room temperature. The reaction mixture was prepared as follows, allowing 75 μl for each slide: DDW, 62.25 μl; 10xTdT buffer, 7.5 μl; 5.25 μl biotin-4-dUTP stock (Sigma; 0.5 mM in 10 mM Tris-HCl); and 1.5 μl TdT (10 units/μl; Boehringer Mannheim). The sections were exposed to reaction mixture and then carefully covered with a glass cover slip and incubated at 37°C for 1-1.5 h in a humid chamber. The reaction was terminated by immersion of the slides in 2xSSC at room temperature for 15 min. Following two further washes in DDW and PBS (pH 7.4), the sections were covered in 3% SM in PBS with 0.5% Tween 20 for 15 min to block non-specific binding. Following this, excess SM was removed and sections were incubated with Extra-avidin-peroxidase® (Sigma), diluted 1:50 in 3% SM in PBS with Tween 20, for 30 min at room temperature. Slides were then washed in PBS, and sections were stained using the AEC procedure (Pierce). Stock AEC solution was prepared by dissolving AEC in DMF to a final concentration of 4 mg/ml. Prior to the color reaction, 0.67 ml of AEC stock was added to 10 ml of 0.1 M sodium acetate buffer, pH 5.2, and 10 μl of 30% H_2O_2 was then added. This solution was filtered and applied to the sections and after 20 min the color reaction was complete. The slides were then washed in PBS and mounted in Crystal Mount® (Fisher Scientific Inc.). Positively stained nuclei were colored intensely red. In later experiments, TUNEL was performed using biotin-16-dUTP (Sigma). This was available at a concentration of 0.3 mM, therefore the volumes in the reaction mixture were modified accordingly. Identical results as with biotin-4-dUTP were obtained with biotin-16-dUTP.

In addition, both positive and negative controls were performed. In the negative controls either the bio-4-dUTP stock or the TdT was omitted from the reaction mixture. For positive controls, sections were treated with DNase 1 buffer (30 mM Trizma base, 140 mM sodium cacodylate, 4 mM magnesium chloride, 0.1 mM dithiothreitol) for 5 min following the quenching of endogenous peroxidase, incubated with fresh DNase 1 (1 μg/ml; Boehringer Mannheim) in buffer for 10 min at room temperature, and then washed in DDW. Processing of the sections was then continued as described above. In negative control slides, no staining was observed in any of the nuclei and the light pink background color was identical to that observed in experimental sections. In positive control sections treated with DNAse 1, every nucleus was stained intensely red (not shown).

Examination of sections for TUNEL-positive cells and TNFα-CRP expression

In order to investigate whether a correlation might exist between the expression of TNFα-CRPs and the presence of programmed cell death in particular tissues, TUNEL was carried out on sections from a region closely adjacent to those which had been stained with the anti-TNFα antibodies immunocytochemically. These sections were examined and correlations were noted between the sites of expression of TNFα-CRPs and TUNEL-positive cells in specific tissues. The results obtained were shown to be consistent by the use of sections from 3-5 different embryos at each stage of development studied, thus ensuring that no abnormal embryos were included in the study.

TUNEL/anti-TNFα double labeling

A double labeling procedure was also used to investigate possible correlations between the expression of TNFα-CRPs and the presence of DNA strand breaks indicative of programmed cell death, detected by TUNEL. Using the monoclonal antibodies, sections that had been previously processed for TUNEL were immunostained using the AS/AP® kit. However, the chromogenic substrate supplied in the kit was replaced by BCIP/NBT (BioRad) so that immunostaining by TNFα antibodies could be visualized as a light blue color, which contrasted well with the intensely stained red nuclei that were detected by TUNEL. Immersion of sections in SSC buffer, and subsequent digestion with proteinase K did not affect the TNFα immunostaining.

Preparation of embryos for the L929-8 TNF bioassay

White leghorn hens' eggs were incubated at 37°C for 3 days (stage 18; Hamburger and Hamilton, 1951). Fifty embryos were removed from their yolk and rinsed and handled in sterile millipore-filtered PBS (pH 7.4) and kept on ice. Embryos were then homogenized in sterile millipore-filtered PBS (pH 7.4) to give a final volume of 3000 μl. Thus, the final homogenate was at a dilution of 1:9. This homogenate was then used immediately in the bioassay.

The L929-8 TNF bioassay

The L929-8 TNF cytolytic bioassay was used to determine the concentration of TNFα-like activity in stage-18 embryos and the percent viability of L929-8 cells in the presence of embryo homogenate with and without neutralizing antibodies to mouse TNFα. L929-8 cells are a highly TNFα-sensitive subclone of the murine fibroblastoid cell line L929 (Branch *et al.*, 1991). L929-8 cells were washed and plated at a density of 5×10^4 cells/50 μl/well in Iscove's modified Dulbecco's medium (Gibco) containing 10% v/v fetal bovine serum supplemented with 2 μg/ml actinomycin D (Sigma) in 96-well tissue culture flat-bottom plates. After 1 h of pre-incubation at 37°C, serial dilutions of chick embryo homogenate (50 μl) were added to the L929-8 cells. Recombinant murine TNFα (1.2×10^7 U/mg, Genentech) standards were included in each assay. Plates were then incubated for 18 h at 37°C and then 50 μl of 0.05% (w/v) neutral red (Sigma) in normal saline was added to each well to stain viable cells. Plates were then incubated for a further 2 h at 37°C, the media was decanted, the plates were washed in PBS and 100 μl of 50% ethanol in 0.05 M sodium phosphate was added. The optical density was determined spectrophotometrically (reference wavelength= 650 nm; sample wavelength= 450 nm). Concentrations of proteins with TNFα-like activity could then be calculated from the standard curve. Each experiment was repeated three times, and similar results were obtained in each case. Confirmation of the L929-8 cell lytic activity in the homogenate as TNFα-like was obtained by pre-incubation of homogenate in the presence of 50 μl of one of two polyclonal neutralizing antibodies to mouse TNFα (Ab1: anti-mouse TNFα, Genzyme; or Ab2: anti-mouse TNFα, Genentech) at a dilution of 1:500. Percentage viabilities of L929-8 cells at particular serial dilutions of embryo homogenate, with and without antibody, were calculated using the following formula:

$$\% \text{ viability} = \frac{OD_{sample} - OD_{100\% \text{ dead}}}{OD_{100\% \text{ viable}} - OD_{100\% \text{ dead}}}$$

Where, OD_{sample}= average optical density from samples of homogenate at a particular dilution; $OD_{100\% \text{ dead}}$= average optical density of samples at 5pg/ml exogenous TNFα (100% killing of L929-8 cells); $OD_{100\% \text{ viable}}$= average optical density of triplicate samples at 0.01pg/ml exogenous TNFα (100% survival of L929-8 cells).

Acknowledgments

We thank Dr. S-M. Ben-Sasson, The Hebrew University, Jerusalem, Israel and Dr. J.P. MacManus, National Research Council, Ottawa, Canada for their help and encouragement with the TUNEL protocol and we thank Dr. L. Guilbert and Dr. K. Bagnall for useful discussion and Ninghe Hu for help with the embryo dissections. This work was supported by an operating grant to E.J.S. from the Medical Research Council of Canada and a studentship awarded to M.A.W. from the Alberta Heritage Foundation for Medical Research.

References

ANSARI, B., COATES, P.J., GREENSTEIN, B.D. and HALL, P.A. (1993). *In situ* end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. *J. Pathol. 170:* 1-8.

BARINAGA, M. (1993). Death gives birth to the nervous system. But how? *Science 259:* 762-763.

BAZAN, J.F. (1993). Emerging families of cytokines and receptors. *Curr. Biol. 3:* 603-606.

BELLAIRS, R. (1961). Cell death in chick embryos as studied by electron microscopy. *J. Anat. 95:* 54-60.

BRANCH, D.R., SHAH, A. and GUILBERT, L.R. (1991). A specific and reliable bioassay for the detection of femtomolar levels of human and murine tumor necrosis factors. *J. Immunol. Methods 143:* 251-261.

BUTTYAN, R., OLSSON, C.A., PINTAR, J., CHANG, C., BANDYK, M., PO-YING, N. and SAWCZUC, I.S. (1989). Induction of the TRPM-2 gene in cells undergoing programmed cell death. *Mol. Cell. Biol. 9:* 3473-3481.

CHU-WANG, I-W. and OPPENHEIM, R.W. (1978). Cell death in the chick embryo spinal cord. 1. A light and electron microscope study of naturally occurring and induced cell loss during development. *J. Comp. Neurol. 177:* 33-57.

COELHO, C.N.D., UPHOLT, W.B. and KOSHER, R.A. (1993). The expression pattern of the chicken homeobox containing gene GHox-7 in developing polydactylous limb buds suggests its involvement in apical ectodermal ridge-directed outgrowth of limb mesoderm and programmed cell death. *Differentiation 52:* 129-137.

COHEN, G.M., SUN, X.M., SNOWDEN, R.T., DINSDALE, D. and SKILLETER, D.N. (1992). Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J. 286:* 331-334.

COLES, H.S.R., BURNE, J.F. and RAFF, M.C. (1993). Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development 118:* 777-784.

ELLIS, R.E., YUAN, Y. and HORVITZ, H.R. (1991). Mechanisms and functions of cell death. *Annu. Rev. Cell Biol. 7:* 663-698.

FRIEBOVÁ, Z. (1975). Formation of the chick mesonephros 1. General outline of development. *Folia Morphol. 23:* 19-39.

GAVRIELI, Y., SHERMAN, Y. and BEN-SASSON, S.A. (1992). Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell Biol. 119:* 493- 501.

GERSCHENSON, L.E. and ROTELLO, R.J. (1992). Apoptosis: a different type of cell death. *FASEB J. 6:* 2450-2455.

GRAHAM, A., HEYMAN, I. and LUMSDEN, A. (1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development 119:* 233-245.

HAMBURGER, V. and HAMILTON, H.I. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol. 88:* 49-92.

HARRIS, A.J. and McCAIG, C.D. (1984). Motoneuron death and motor unit size during embryonic development of the rat. *J. Neurosci. 4:* 13-24.

HERNÁNDEZ-CASELLES, T. and STUTMAN, O. (1993). Immune functions of tumor necrosis factor. 1. Tumor necrosis factor induces apoptosis of mouse thymocytes and can also stimulate or inhibit IL-6-induced proliferation depending on the concentration of mitogen costimulation. *J. Immunol. 151:* 3999-4012.

HINCHLIFFE, J.R. (1981). Cell death in vertebrate limb morphogenesis. *Prog. Anat. 2:* 1-17.

HOMMA, S., YAGINUMA, H. and OPPENHEIM, R.W. (1994). Programmed cell death during the earliest stages of spinal cord development in the chick embryo: a possible means of early phenotypic selection. *J. Comp. Neurol. 345:* 377-395.

JEFFS, P. and OSMOND, M. (1992). Segmentally regulated patterns of cell death in the hindbrain and trunk of the developing chick embryo. In *Formation and Differentiation of Early Embryonic Mesoderm* (Eds. R. Bellairs, E.J. Sanders and J.W. Lash). Plenum Press, New York, pp. 155-167.

JESSEN, K.R., BRENNAN, A., MORGAN, L., MIRSKY, R., KENT, A., HASHIMOTO, Y. and GAVRILOVIC, J. (1994). The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. *Neuron 12:* 509-527.

JIANG, H. and KOCHHAR, D.M. (1992). Induction of tissue transglutaminase and apoptosis by retinoic acid in the limb bud. *Teratology 46:* 333-340.

KERR, F.R., SEARLE, J., HARMON, B.V. and BISHOP, C.J. (1987). Apoptosis. In *Perspectives in Mammalian Cell Death* (Ed. C.S. Potten). Oxford University Press, Oxford, pp. 93-128.

KOSEKI, C., HERZLINGER, D. and AL-AWQATI, Q. (1992). Apoptosis in metanephric development. *J. Cell Biol. 119:* 1327-1333.

LARRICK, J.W. and WRIGHT, S.C. (1990). Cytotoxic mechanisms of tumor necrosis factor-α. *FASEB J. 4:* 3215-3223.

OPPENHEIM, R.W. (1991). Cell death during development of the nervous system. *Annu. Rev. Neurosci. 14:* 453-501.

PEXIEDER, T. (1975). Cell death in the morphogenesis and teratogenesis of the heart. *Adv. Anat. Embryol. Cell Biol. 51:* 1-100.

RABIZADEH, S., OH, J., ZHONG, L-T., YANG, J., BITLER, C.M., BUTCHER, L.L. and BREDESEN, D.E. (1993). Induction of apoptosis by the low affinity NGF-receptor. *Science 261:* 345-348.

RAFF, M.C., BARRES, B.A., BURNE, J.F., COLES, H.S., ISHIZAKI, Y. and JACOBSEN, M.D. (1993). Programmed cell death and the control of cell survival: lessons from the nervous system. *Science 262:* 695-700.

ROTHE, J., GEHR, G., LOETSCHER, H. and LESLAUER, W. (1992). Tumor necrosis factor receptors — structure and function. *Immunol. Res. 11:* 81-90.

ROWE, D.A., CAIRNS J.M. and FALLON J.F. (1982). Spatial and temporal patterns of cell death in limb bud mesoderm after apical ectodermal ridge removal. *Dev. Biol. 93:* 83-91.

SAUNDERS, J.W., GASSELING, M.T. and SAUNDERS, L.C. (1962). Cellular death in morphogenesis of the avian wing. *Dev. Biol. 5:* 147-178.

SAVILL, J., FADOK, V., HENSEN, P. and HASLETT, C. (1993). Phagocyte recognition of cells undergoing apoptosis. *Immunol. Today 14:* 582-590.

SCHWARTZ, L.M. (1991). The role of cell death genes during development. *BioEssays 13:* 389-395.

SCHWARTZ, L.M. and OSBORNE, B.A. (1993). Programmed cell death, apoptosis and killer genes. *Immunol. Today 14:* 582-590.

SMEYNE, R.J., VENDRELL, M., HAYWARD, M., BAKER, S.J., MIAO, G.G., SCHILLING, K., ROBERTSON, L.M., CURRAN, T. and MORGAN, J.I. (1993). Continuous *c-fos* expression precedes programmed cell death *in vivo*. *Nature 363:* 166-169.

SNOW, M.H.L. (1987). Cell death in embryonic development. In *Perspectives in Mammalian Cell Death* (Ed. C.S. Potten). Oxford University Press, Oxford, pp. 202-228.

TOMEI, L.D., SHAPIRO, J.P. and COPE, F.O. (1993). Apoptosis in C3H/10T$^{1/2}$ mouse embryonic cells: evidence for internucleosomal DNA modification in the absence of double strand cleavage. *Proc. Natl. Acad. Sci. USA 90:* 853-857.

VASSALLI, P. (1992). The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol. 10:* 411-452.

VAUX, D.L. (1993). Towards an understanding of the molecular mechanisms of physiological cell death . *Proc. Natl. Acad. Sci. USA 90:* 786-789.

WIJSMAN, J.H., JONKER, R.R., KEIJZER, R., VAN DE VELDE, C.J.H., CORNELL ISE, C.J. and VAN DIERENDONCK, J.H. (1993). A new method to detect apoptosis in paraffin sections: *in situ* end-labelling of fragmented DNA. *J. Histochem. Cytochem. 41:* 7-12.

WRIDE, M.A. and SANDERS, E.J. (1993a). Expression of tumor necrosis factor-α (TNFα)-cross-reactive proteins during early chick embryo development. *Dev. Dynamics* 198: 225-239.

WRIDE, M.A. and SANDERS, E.J. (1993b). Programmed cell death in chick embryo development: a study using TdT-mediated dUTP-biotin nick end labeling (TUNEL) and correlation with expression of TNFα cross-reactive proteins. *Mol. Biol. Cell 4s:* 143a.

WRIDE, M.A. and SANDERS, E.J. (1994). Potential roles for tumour necrosis factorα during embryonic development. *Anat. Embryol.* (In press).

WRIGHT, S.C., KUMAR, P., TAM, A.W., SHEN, N., VARMA, M. and LARRICK, J.W. (1992). Apoptosis and DNA fragmentation precede TNF-induced cytolysis in U937 cells. *J. Cell. Biochem. 48:* 344-355.

YAMASU, K., ONOE, H., SOMA, G.I., OSHIMA, H. and MIZUNO, D.I. (1989). Secretion of tumor necrosis factor during fetal and neonatal development of the mouse: ontogenic inflammation. *J. Biol. Response Mod. 8:* 644-655.

ZAKERI, Z.F., QUAGLINO, D., LATHAM, T. and LOCKSHIN, R.A. (1993). Delayed internucleosomal DNA fragmentation in programmed cell death. *FASEB. J. 7:* 470-478.

Accepted for publication: September 1994