



# Cell death in the gastrulating chick embryo: potential roles for tumor necrosis factor- $\alpha$ (TNF- $\alpha$ )

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Received 31.5.96; revised 12.08.96; accepted 4.10.96

Edited by C.J.Thiele

## Abstract

We have examined the expression of TNF- $\alpha$  and its receptors, TNFR1 and TNFR2, during gastrulation in the chick embryo, and have investigated the possible role of this factor in the control of cell death at this early stage of development. TNF- $\alpha$ , immunoreactive at approximately 17 kD, was found both *in vivo* and *in vitro*, most intensely associated with the cell surface and cytoskeleton of endoderm cells. TNFR2 was especially immunoreactive in endoderm cells of the marginal zone. TNFR1 was found in nuclei throughout the embryo. Embryos also showed widespread expression of both the *bcl-2* and *Bax* gene products, which are both associated with cell death pathways. Intact embryos in culture were sensitive to the addition of TNF- $\alpha$  (approx. 110 ng/ml), responding by significantly increasing the incidence of DNA fragmentation in cells from all tissues of the embryo. This effect was abrogated by immunological pre-absorption of the cytokine. Cultured cells from these embryos also responded to the addition of agonistic antibodies to TNF- $\alpha$  receptors by increasing DNA fragmentation. A similar response to TNF- $\alpha$  antiserum by cultured cells appeared to be related to a concomitant decrease in cell-substratum adhesion caused by the antibody. Decreased cell adhesion, induced non-specifically with anti-integrin antiserum, also resulted in increased DNA fragmentation. TNF- $\alpha$ , synthesized and secreted by the embryo itself, may be able to exert a paracrine effect on the incidence of cell death in tissues of the embryo, and the cell death process may be related to the expression of *bcl-2* and *Bax* gene products. The influence of TNF- $\alpha$  may be exerted by the activation of cell death signalling pathways directly, or indirectly through perturbation of the cytoskeleton or of integrin-mediated cell adhesion.

**Keywords:** chick, embryo, gastrulation, cell death, TNF- $\alpha$

**Abbreviations:** AEC, 3-amino-9-ethyl carbazole; BrdU, Bromodeoxyuridine; BSA, Bovine serum albumin; DDW, Double distilled water; ICE, Interleukin-1 $\beta$ -converting en-

zyme; IFN-gamma, Interferon-gamma; ITS, Insulin, Transferin, Selenium; PBS, Phosphate buffered saline; SM, Skimmed milk; TNF- $\alpha$ , Tumor necrosis factor- $\alpha$ ; TNFR1, Tumor necrosis factor receptor 1; TNFR2, Tumor necrosis factor receptor 2; TUNEL, terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end-labelling; UTP, Uridine triphosphate

## Introduction

It has been recognized for many years that cell death during embryonic development is a significant contributor to the process of morphogenesis (Glücksmann, 1951; Saunders, 1966; Snow, 1987; Sanders and Wride, 1995). Some well-defined sites where the importance of this cell death has been particularly widely acknowledged include: the limb bud (Hinchliffe, 1981); the heart (Pexieder, 1975); and the palate (Ferguson, 1988). However, it has also been noted that cell death is a feature of morphogenesis at much earlier stages of development, such as during gastrulation (Bellairs, 1961), neural tube closure (Schlüter, 1973), and neural crest formation (Jefferis *et al*, 1992; Homma *et al*, 1994). At these early times in development, the extent and patterns of cell death are very much less clear, and its significance is essentially unknown.

Data on cell death in the pre-streak mammalian embryo suggests that deaths are widespread throughout blastocyst development of a number of species (Carnegie *et al*, 1985; Hardy *et al*, 1989; Enders *et al*, 1990), and may reach levels as high as 10% in the inner cell mass (El-Shershaby and Hinchliffe, 1974; Copp, 1978; Pierce *et al*, 1989). During mammalian gastrulation, significant numbers of cell deaths occur (Daniel and Olson, 1966; Poelmann and Vermeij-Keers, 1976; Lawson *et al*, 1986; Sanders *et al*, 1996) which may amount to some 15–20% of the total cell population.

In the chick embryo, where the process of gastrulation is more accessible to experimentation, cell death with apoptotic morphology is widespread (Bellairs, 1961), and recent detailed analysis (Sanders *et al*, 1996) indicates that it occurs in all germ layers throughout the embryo. There is a suggestion that cell death at this time is associated with the differentiation of the presumptive neural plate (Jacobson, 1938), but generally the relationship with morphogenesis is unclear.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a multifunctional 17 kDa peptide with well-established pathophysiological roles (Vassalli, 1992; Tracey and Cerami, 1994), including the ability to precipitate DNA fragmentation (Larrick and Wright, 1990; Wright *et al*, 1992), which is one of the main early characteristics of apoptotic cell death (Bortner *et al*, 1995). TNF- $\alpha$  exerts its effects through the activity of two distinct receptors, TNFR1 of 55–60 kDa and TNFR2 of 75–80 kDa (Tartaglia *et al*, 1991), one or both of which are

necessary for induction of apoptosis (Smith *et al*, 1994; Vandenabeele *et al*, 1995).

Because of the association of TNF- $\alpha$  with cell death, and because of the significance of cell death to developmental processes, we have previously speculated on possible developmental roles for TNF- $\alpha$  (Wride and Sanders, 1995). There is a growing body of literature to indicate that TNF- $\alpha$  mRNA and protein are widely expressed during the embryogenesis of the chick, mouse and human (Ohsawa and Natori, 1989; Gendron *et al*, 1991; Lachapelle *et al*, 1993; Hunt *et al*, 1993; Kohchi *et al*, 1994). In particular, TNF- $\alpha$  has been localized in gastrulating embryos (Wride and Sanders, 1993), and in several tissues during the next few days of development, including: myotome, neural tissue, notochord, lens, and lung (Wride and Sanders, 1993; Jaskoll *et al*, 1994). Further, during this period it is bioactive in eliciting cell death (Wride *et al*, 1994). Indeed, TNF- $\alpha$  appears to be one member of an array of cytokines present during early development that includes interleukins and interferon (Rothstein *et al*, 1992).

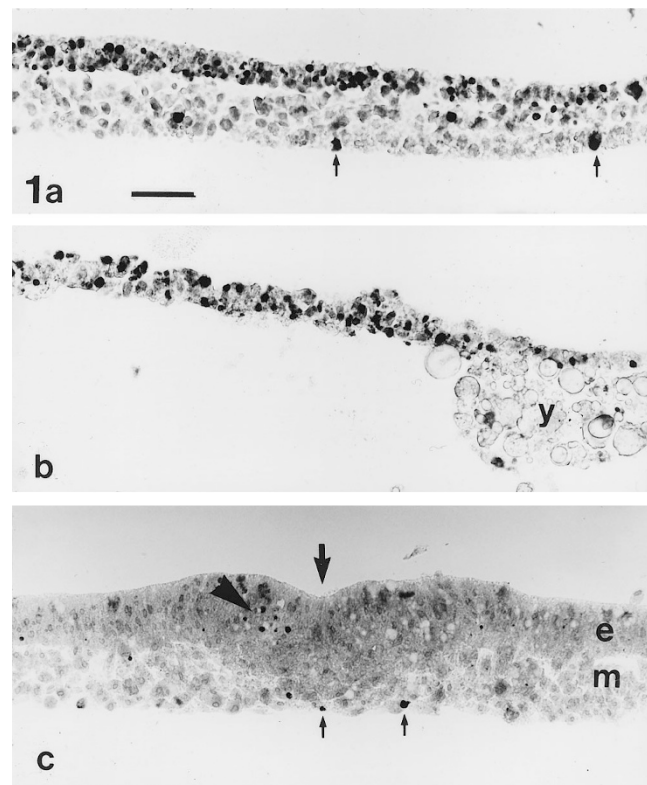
To what extent TNF- $\alpha$  has significant physiological roles during development remains unclear. Gene 'knockout' experiments (reviewed by Beutler, 1995), in which the receptors for TNF- $\alpha$  are deleted separately or in combination have yielded no gross developmental anomalies. Knockout of the TNF- $\alpha$  gene simultaneously with the lymphotoxin- $\alpha$  (TNF- $\beta$ ) gene results in developmental perturbation in the lymphoid system, but since knockout of lymphotoxin- $\alpha$  alone causes the same anomaly, this experiment does not provide evidence for a role for TNF- $\alpha$  in development. Similarly, a transgenic mouse line constitutively expressing an inhibitor of TNF- $\alpha$  and TNF- $\beta$  failed to produce anomalous phenotypes (Peppel *et al*, 1993). On the other hand, repeated injection of TNF- $\alpha$  antibodies into pregnant mice produces growth retardation and the development of abnormal lymphoid organs in the embryos (de Kossodo *et al*, 1992). Further, Vassalli (1993) has argued that the results of the TNF-receptor knockout experiments may not provide definitive evidence for roles for TNF- $\alpha$  itself, citing as confounding factors not only redundancy, but also the possibility of functions that are not mediated by the known receptors.

We have previously noted spatio-temporal correlations in the distribution of TNF- $\alpha$ -related proteins and programmed cell death in the chick embryo (Wride *et al*, 1994). In the present work, we seek to substantiate a relationship between this factor and cell death in development, using the early chick embryo as a model. Since there is evidence that DNA fragmentation is an early event in programmed cell death during chick development (Toné *et al*, 1994), we assess cell death using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end-labelling (TUNEL) technique (Gavrieli *et al*, 1992; Wijsman *et al*, 1993). We give an account of the expression of TNF- $\alpha$  and its receptors during gastrulation, and take advantage of the accessibility of the chick embryo to provide experimental evidence for a role for this factor in the cell death that is occurring at this time in development.

## Results

### Cell death in the gastrulating embryo

We have recently carried out a detailed analysis of the distribution of nuclei undergoing DNA fragmentation and cell death during this developmental period in chick and mouse embryos (Sanders *et al*, 1996). Here, we summarize the situation for the chick in the context of the present work. Dying cells are apparent in all three germ layers of the embryo at this time, with particularly high numbers occurring in the epiblast. Especially notable was an arc of extensive cell death in the epiblast located in the rostral region of the embryo corresponding to the 'germinal crescent' (Figure 1a), and in the peripheral 'marginal zone', at the boundary between the area pellucida and the area opaca (Figure 1b). The primitive streak itself, and the tissues to either side of it, were generally regions of relatively low cell death, although local areas of higher cell death in the primitive streak could be found (Figure 1c).



**Figure 1** (a) Transverse section through the rostral region of the area pellucida of a stage 5 embryo, processed for the TUNEL technique to demonstrate nuclei undergoing DNA fragmentation. Positive nuclei are densely stained. In this region of the embryo, DNA fragmentation is extensive in nuclei of the upper epiblast layer, and scattered in nuclei in the middle mesoderm layer and the lower endoderm layer (arrows). Bar=50  $\mu$ m in Figures 1–3. (b) As a, but through the peripheral region of the area pellucida, where it abuts the yolk area opaca (the marginal zone). TUNEL-positive nuclei are abundant in this region. y=yolk. (c) As a, but through the primitive streak (large arrow). Nuclei showing DNA fragmentation are sparse in this region, although clusters of positive nuclei are seen in the streak itself (arrowhead). Only scattered positive nuclei are found throughout this region of the embryo, including the lower endoderm layer (small arrows). e=epiblast; m=mesoderm.

### Western blotting for TNF- $\alpha$

We have previously shown that immunoblotting chick embryo homogenate with antibodies against mouse TNF- $\alpha$  gives rise to bands at a number of different molecular weights, notably 50 kDa and 70 kDa (Wride and Sanders, 1993). Using the modified methods described, and the polyclonal antibody, we now demonstrate a band at approximately 17 kDa in homogenate of gastrulating embryos (Figure 2), corresponding to the molecular weight of native TNF- $\alpha$ , in addition to the higher molecular weight forms that we described previously.

This method was also used to confirm that there was no detectable TNF- $\alpha$  present in the fetal bovine serum used for *in vitro* culture of cells (result not shown).

### Immunocytochemistry for TNF- $\alpha$ , TNFR1 and TNFR2 in embryos

During gastrulation, TNF- $\alpha$  was localized most intensely to the endoderm layer of cells (Figure 3a; monoclonal MP6 XT22) across the entire embryo, including the region immediately below the primitive streak. TNFR1 was localized to nuclei in cells of all layers of the embryo (Figure 3b), while TNFR2 was found primarily in endoderm layer (Figure 3c). The distribution of TNFR2 was not uniform in this layer, however, being present in the cytoplasm or on the surfaces of individual cells scattered within the endoderm. Immunoreactivity was particularly intense in endoderm cells at the lateral edges of the embryo where the endoderm approached the marginal zone (Figure 3d).

At stages of development earlier than gastrulation, these three antigens were detected only diffusely throughout the



**Figure 2** Western blot, showing recombinant TNF- $\alpha$  running at 17 kDa (lane 1). In whole homogenate of stage 5 embryos (lane 2) immunoreactive bands are found at approximately 17 kDa, as well as at 70 kDa.

embryo, with TNFR1 again showing a preferential nuclear localization (not shown).

We have previously described the distribution of TNF- $\alpha$  in several tissues at later stages of development (Wride and Sanders, 1993). With regard to the receptor distributions, we found here that the immunoreactivity of both TNFR1 and TNFR2 declines immediately after gastrulation, with TNFR1 reoccurring first in well-defined locations, most notably in the myotome.

### Immunocytochemistry for *bcl-2*, *Bax* and *ICE*

Because the *bcl-2* and *Bax* gene products have been closely linked to the regulation of apoptotic cell death, often functioning through the action of interleukin-1 $\beta$ -converting enzyme (ICE; Hockenbery, 1995), embryos were examined for the occurrence of these proteins. Sections of embryos immunostained for these antigens showed widespread expression of both the *bcl-2* and *Bax* gene products in all tissues. (Figure 3e, f, g). Immunoreactivity for ICE was not detected.

### Cell death in cultured cells

When cultures of cells from the epiblast, mesoderm and endoderm of gastrulating embryos were examined for DNA fragmentation after 1 day in serum-free culture, very few cells were found to be TUNEL-positive (Figure 4a; Table 2). A correlation was noted, however, between cell detachment from the substratum and increased incidence of DNA fragmentation (Figure 4b).

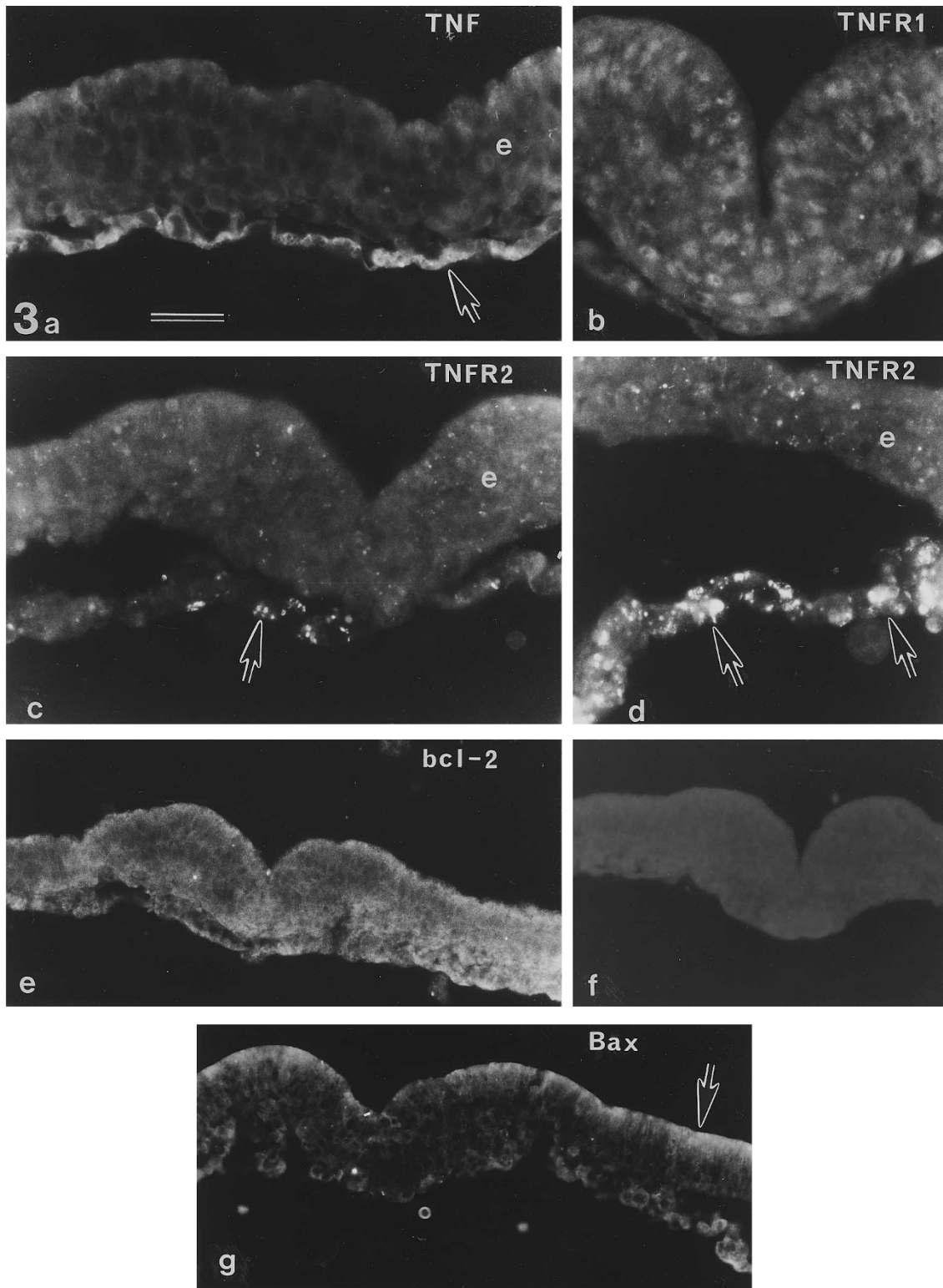
### Immunocytochemistry for TNF- $\alpha$ , TNFR1 and TNFR2 in cultured cells

Localization of TNF- $\alpha$  to cytoskeletal stress fibres was found in cells from all of the three tissues using the monoclonal antibody MP6-XT22 (Figure 4c), and this was not affected by pre-absorption of the antibody with actin. Cell surfaces were immunoreactive, most noticeably with the polyclonal antibody (Figure 4d).

As in the sectioned material, TNFR1 was found to be associated with cell nuclei, and this relationship was strongest for cells of the endoderm (Figure 4e). TNFR2 distribution also supported the results on sectioned material, being localized to the cytoplasm in microfilaments (Figure 4f). This result was not affected by pre-absorption of the antibody with actin.

### Effects of TNF- $\alpha$ on cell death in embryos in culture (Table 1)

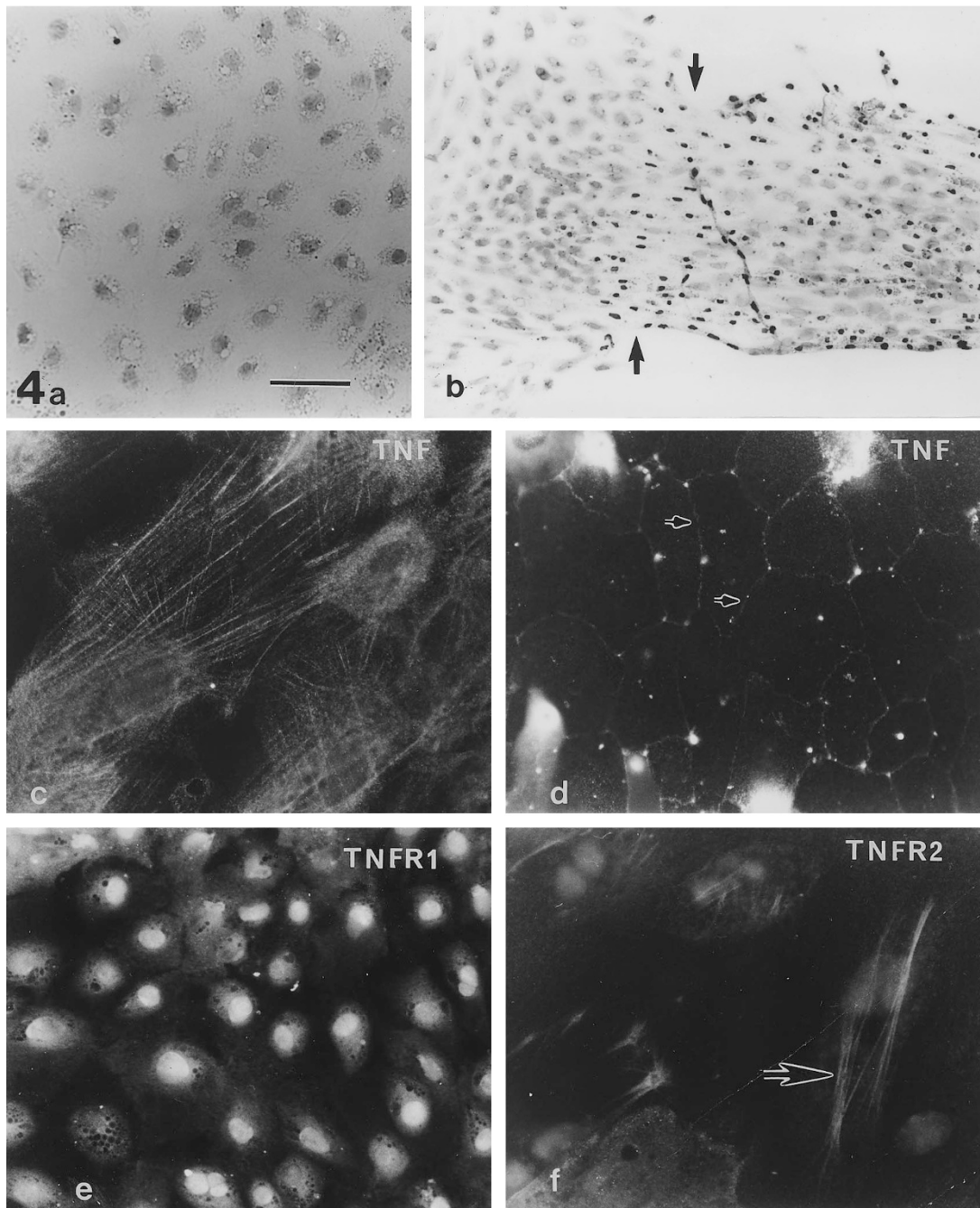
Embryos cultured by the method of New (1955) for 6 h showed levels of DNA fragmentation which were similar to that observed in embryos of the same age taken directly from the egg. The addition of 10  $\mu$ l of saline to the exposed surface of the embryo during the incubation period did not change the incidence of TUNEL-positive nuclei (Figure 5a), which, in the region of the primitive streak, remained at very low levels. The addition of TNF- $\alpha$  at concentrations from 100–400 ng/ml for



**Figure 3** (a) Localization of TNF $\alpha$ , showing intense immunoreactivity in the lower endoderm layer of cells (arrow). e=epiblast. Bar=50  $\mu$ m. (b) Localization of TNFR1, showing the nuclear localization of this receptor. Bar=50  $\mu$ m. (c) Localization of TNFR2, showing immunoreactivity in the endoderm close to the primitive streak (arrow). e=epiblast. Bar=50  $\mu$ m. (d) Localization of TNFR2, showing intense immunoreactivity in the endoderm at the periphery of the area pellucida, in the marginal zone (arrows). e=epiblast. Bar=50  $\mu$ m. (e) Immunoreactivity for *bcl-2* shows widespread distribution of this gene product throughout the embryo. Bar=100  $\mu$ m. (f) Control section, in which the antibody to *bcl-2* was pre-absorbed with *bcl-2* peptide. There is no labelling. Bar=100  $\mu$ m. (g) Immunoreactivity for *Bax* shows labelling throughout the embryo, but concentrated at the apical ends of the epiblast cells (arrow). Bar=100  $\mu$ m.

8 h also had no effect, however at 500 ng/ml for 8 h a highly significant increase in the incidence positive nuclei was observed (Table 1; Figure 5b). This increase was observed in all three tissue layers of the embryo, and in all regions of the

blastoderm, but was not catastrophic in that no reproducible deleterious effect on development could be observed after treatment overnight ( $n=10$ ). This promotion of DNA fragmentation could be abolished by pre-absorption of the TNF- $\alpha$  with



**Figure 4** (a) A 2-day culture of untreated endoderm cells, showing that the nuclei of these cells are TUNEL-negative under these conditions. Bar=100  $\mu$ m. (b) As Figure (a), except that this untreated endoderm culture has partially detached from the substratum at the arrows. The part of the culture to the left is adherent to the substratum, and TUNEL-negative; the part of the culture to the right has partially detached, and is TUNEL-positive. Bar=400  $\mu$ m. (c) Localization of TNF $\alpha$  using monoclonal antibody in endoderm cultures shows immunoreactivity associated with the cytoskeleton. Bar=50  $\mu$ m. (d) Localization of TNF $\alpha$  using polyclonal antibody in endoderm cultures shows association with cell surfaces (arrows). Bar=100  $\mu$ m. (e) Localization of TNFR1 in endoderm cultures shows a nuclear localization of this receptor. Bar=100  $\mu$ m. (f) Localization of TNFR2 in endoderm cultures shows association of this receptor with the cytoskeleton (arrow). Bar=50  $\mu$ m.

**Table 1** Effects of TNF $\alpha$  on DNA fragmentation in intact embryos in culture

Treatment (8 h)	DNA Fragmentation
Saline	1.30 $\pm$ 1.17
TNF $\alpha$ (100 ng/ml)	1.19 $\pm$ 0.72
TNF $\alpha$ (400 ng/ml)	0.74 $\pm$ 0.85
TNF $\alpha$ (500 ng/ml)	6.16 $\pm$ 4.15**
Anti-TNF $\alpha$ PAb	1.61 $\pm$ 0.96
TNF $\alpha$ pre-absorbed (500 ng/ml)	1.45 $\pm$ 0.56

TUNEL-positive nuclei were scored in sections of embryos that had been cultured and treated with TNF $\alpha$  and its polyclonal antibody. The concentrations of TNF $\alpha$  shown were the initial concentrations applied. These concentrations were reduced during culture, by dilution, so that the effective concentration was reduced from 500 ng/ml to approximately 110 ng/ml. \*\*Significantly different from saline,  $P < 0.01$ .

**Table 2** Analysis of cell cultures for TUNEL-positive nuclei

Treatment (8 h)	Endoderm	Mesoderm	Epiblast
ITS	0.12 $\pm$ 0.05	0.04 $\pm$ 0.03	0.06 $\pm$ 0.06
TNF $\alpha$ (100 ng/ml)	0.21 $\pm$ 0.21	0.30 $\pm$ 0.16	0.22 $\pm$ 0.25
TNF $\alpha$ (500 ng/ml)	0.16 $\pm$ 0.13	0.68 $\pm$ 0.85	0.20 $\pm$ 0.15
anti-TNF $\alpha$ MAb1	9.66 $\pm$ 5.58**†	0.60 $\pm$ 0.65*†	0.65 $\pm$ .31*†
anti-TNF $\alpha$ MAb2	2.29 $\pm$ 1.57*†	2.90 $\pm$ 1.90*†	1.45 $\pm$ 0.97*†
anti-TNF $\alpha$ PAb	0.69 $\pm$ 0.75†	1.01 $\pm$ 0.28*†	0.88 $\pm$ 0.37*†
anti-TNF r1	4.49 $\pm$ 2.77**	5.32 $\pm$ 4.2**	0.45 $\pm$ 0.15*
anti-TNF r2	9.84 $\pm$ 2.30**	8.38 $\pm$ 2.56**	6.83 $\pm$ 1.40**

In cultured tissues after treatment of the cultures with TNF $\alpha$  and various antibodies to TNF $\alpha$ . ITS<sup>TM</sup>=defined control medium containing insulin, transferrin and selenium. Anti-TNF $\alpha$  MAb1=clone MP6-XT22 (Endogen/AbV); Anti-TNF $\alpha$  MAb2=clone MP8-XT22 (UBI); \*\*=Significantly different from ITS,  $P < 0.01$ ; \*=Significantly different from ITS,  $P < 0.05$ ; †=Labelled nuclei at the periphery of the culture; Mouse cells-ITS:0.43  $\pm$  0.11; -TNF $\alpha$  (100 ng/ml):0.56  $\pm$  0.18.

polyclonal antibody (1  $\mu$ l undiluted antiserum plus 50  $\mu$ l TNF- $\alpha$ , 500 ng/ml), while the antibody alone at the same dilution was without effect (Table 1).

Measurements of the accumulation of fluid on the surface of embryos in culture indicated that during the 8 h incubation period, the test solution was being progressively diluted. The volume of fluid accumulated in this time was observed to be 34.4  $\mu$ l ( $\pm$ 8.1;  $n=10$ ), meaning that during the incubation period, the TNF- $\alpha$  concentration fell from 500 ng/ml to 110 ng/ml.

### Effects on cell death in cultured cells (Table 2)

In contrast to the effects in embryo culture, TNF- $\alpha$ , in a concentration series from 10–500 ng/ml had no effect on cell death in *in vitro* cultures of cells from any of the embryonic layers. Cultures were allowed to establish for 6 h before addition of the factor, and treatment was continued for varying periods up to 3 days before the cultures were assayed by the TUNEL method. In these experiments the cultures after treatment showed the same low levels of TUNEL-positive cells as in the controls (Figure 4a). In order to determine if this lack of effect was due to a species specific failure of mouse TNF- $\alpha$  to act on chick cells, this experiment was repeated using cultures of cells from mouse embryos at an equivalent stage of development. TNF- $\alpha$  was equally ineffective in eliciting DNA fragmentation in mouse cell cultures.

By contrast, monoclonal and polyclonal antibodies against TNF- $\alpha$  at 1:10 dilution caused statistically significant increases in the incidence of cell death, primarily affecting cells at the periphery of the culture (Figure 5c). We have concluded that this non-uniform distribution of dying cells is related to effects of these antibodies on the detachment of marginal cells from the substratum (see below).

The addition of agonistic antibodies against TNFR1 and TNFR2 (1:150 and 1:120, respectively) resulted in highly significant increases in the occurrence of TUNEL-positive nuclei distributed throughout the cultures (Table 2). Of the two antibodies, the one against TNFR2 was the more effective (Figure 5d).

### Effects on cell proliferation in cultured cells

In view of a recent report showing that TNF- $\alpha$  can stimulate cell proliferation in mouse primordial germ cells (Kawase *et al.* 1994), we tested for this effect in cultures of epiblast, mesoderm and endoderm cells. Although a substantial proportion of cells from each of the embryonic layers incorporated the BrdU label in untreated cultures (Figure 5e), there was no significant effect of TNF- $\alpha$  or its antibodies on the rate of cell proliferation in these cultures. Quantification of proliferation was carried out and analyzed as for the TUNEL cultures.

### Relationship between cell adhesion and cell death

In order to test the idea that a disruption in cell-to-substratum adhesion *in vitro* is able to induce DNA fragmentation in these cells, we cultured endoderm cells on a fibronectin substratum and treated the cultures with V<sub>2</sub>E<sub>9</sub> monoclonal antibody to chicken integrin  $\beta_1$  complex. Cultures were established in normal medium overnight, and then treated with supernatant overnight before fixing and taking through the TUNEL procedure. The treated cultures showed a significantly ( $P < 0.01$ ) higher incidence of nuclei showing DNA fragmentation than controls cultured in the presence of non-specific mouse IgG (0.07  $\pm$  0.06,  $n=10$ ; vs. 0.42  $\pm$  0.21,  $n=10$ ).

### Discussion

Although it is difficult to know if internucleosomal DNA fragmentation is invariably a feature of programmed cell death in development, it is generally regarded as a reliable, if not necessarily universal, characteristic of apoptosis (Compton, 1992; Bortner *et al.*, 1995), so that use of the TUNEL technique as a marker for dying cells is an appropriate approach. Furthermore, in the early chick embryo, DNA fragmentation has been demonstrated in tissues known to be undergoing programmed cell death (Toné *et al.*, 1994; Wride *et al.*, 1994); cells die with the morphological features of apoptosis (Bellairs, 1961); and cells show antigenic determinants characteristic of apoptotic cells (Fernandez *et al.*, 1994). Although not all programmed cell death occurs by apoptosis, evidence for non-apoptotic death in the early embryo is meagre (Sanders and Wride, 1995). Further, while DNA fragmentation also occurs in dividing cells, the levels of nick-

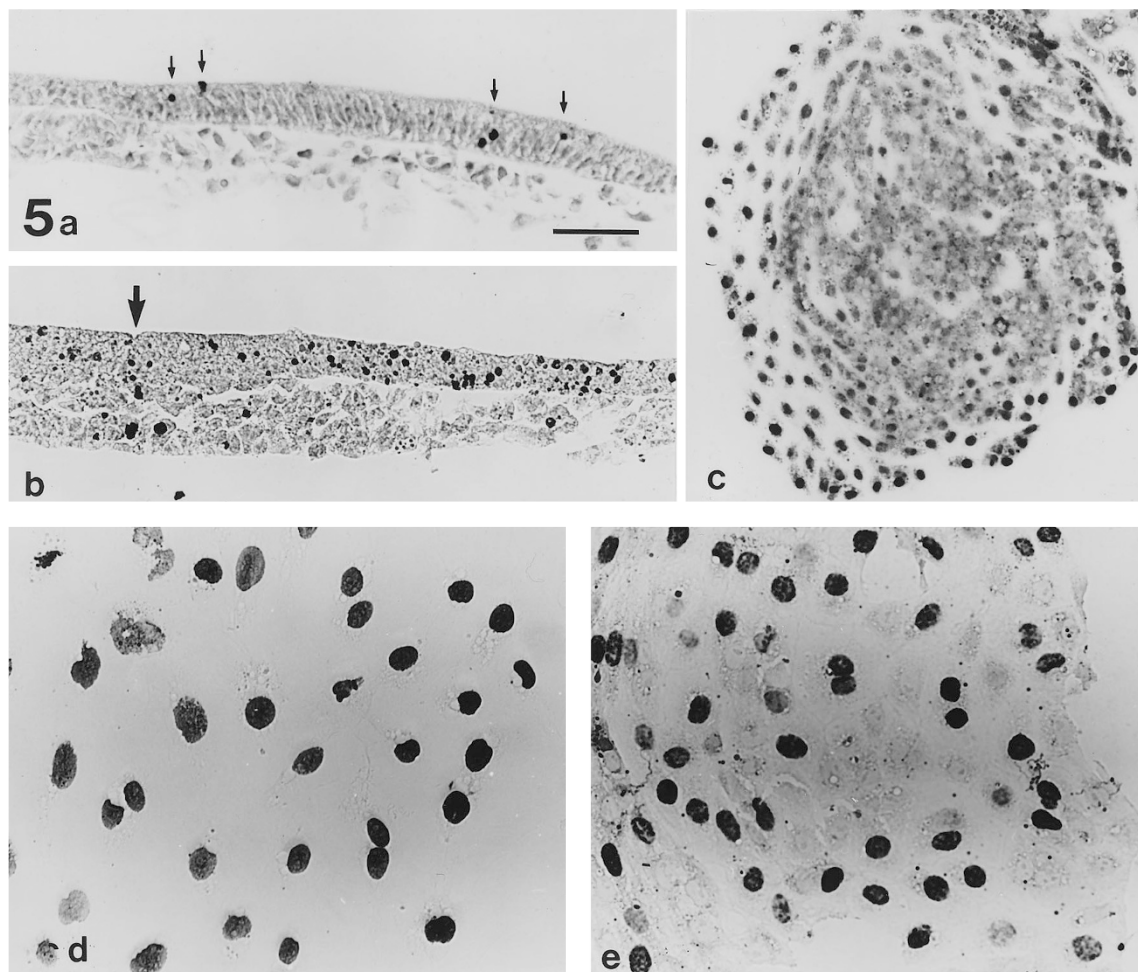
end-labelling in these circumstances is comparatively low and does not obscure the relationship between fragmentation and cell death (Gold *et al*, 1993).

We have previously reviewed the evidence for a role for TNF- $\alpha$  in several different aspects of development, including cell death (Wride and Sanders, 1995), and we have drawn attention to spatio-temporal correlations between patterns of developmental cell death in the chick embryo and the distribution of TNF- $\alpha$  (Wride *et al*, 1994). Widely expressed in early development, TNF- $\alpha$  has been implicated not only in cell death, but also in muscle differentiation (Miller *et al*, 1988), organ branching morphogenesis (Jaskoll *et al*, 1994) and primordial germ cell proliferation (Kawase *et al*, 1994).

Although little is known of avian TNF- $\alpha$  itself (Qureshi *et al*, 1993; Zhang *et al*, 1995), it is known that this molecule is highly evolutionarily conserved (Ahne, 1994). We have shown earlier that several high molecular weight proteins

present in the chick embryo are immunoreactive against antibodies to murine TNF- $\alpha$  (Wride and Sanders, 1993), and we show here that an approximately 17 kDa immunoreactive protein, closely corresponding to the size of murine TNF- $\alpha$ , is also present in the gastrulating chick embryo.

In the present study, we have shown that during gastrulation TNF- $\alpha$  and both of its receptors are found in the embryo. Whereas TNFR1 was found in cells throughout the embryo with a predominantly nuclear location, both TNF- $\alpha$  and TNFR2 were localized primarily to the endoderm, with this receptor being particularly abundant in cells of the marginal zone. The source of the TNF- $\alpha$  is unknown, so it is not clear if the endoderm is a site of synthesis, or a site of accumulation of TNF- $\alpha$  by the binding action of TNFR2. Examination of cultured cells clearly demonstrated the cell surface binding of TNF- $\alpha$  in all three cell types. The primary site of cell death at this time is the



**Figure 5** (a) Transverse section through a stage 5 embryo, close to the primitive streak, cultured for 6 h in the presence of saline. Few cells are TUNEL-positive (arrows; compare the frequency with that shown in Figure 3). Bar=400  $\mu$ m. (b) As Figure (a), but the embryo was treated with TNF $\alpha$  for 6 h (final concentration approximately 100 ng/ml). There are large numbers of TUNEL-positive cells close to the primitive streak (arrow). Bar=400  $\mu$ m. (c) Endoderm culture treated with antibodies to TNF $\alpha$ . TUNEL-positive cells are found primarily at the periphery of the culture. Bar=400  $\mu$ m. (d) Endoderm culture treated with antagonistic antibodies to TNFR2. A high proportion of cells are TUNEL-positive (see Table 2). Bar=100  $\mu$ m. (e) Endoderm culture treated with BrdU to assess the rate of cell proliferation. Many cells are proliferating, and this was not altered by treatment with TNF $\alpha$ , its receptors, or their antibodies. Bar=100  $\mu$ m.



epiblast, particularly in the marginal zone, with lesser amounts of death in each of the other cell layers (Sanders *et al*, 1996). It seems possible therefore that either the synthesis of TNF- $\alpha$  by the endoderm, or its binding there, acts as a trigger for cell death in cells in other cell layers of the embryo, most particularly in the marginal zone of the epiblast overlying the greatest localization of TNFR2. Since the vast majority of TNF- $\alpha$  produced by cells is secreted into the extracellular compartment (Beutler, 1995), paracrine control of cell death within the embryo is likely. Paracrine control of chick embryo differentiation by TNF- $\alpha$  has been suggested previously by Jaskoll *et al* (1994) in another context, and paracrine control of cell death in the mouse embryo by the HNF-4 transcription factor has also been speculated upon (Chen *et al*, 1994).

The triggering of DNA fragmentation in nuclei from all cell layers by application of exogenous TNF- $\alpha$  (at approximately 110 ng/ml) in embryo cultures, indicates the capacity of many cells to respond to this factor, supporting the contention that TNF- $\alpha$  may be a physiological regulator of cell death in the embryo. TNF- $\alpha$ -like killing activity is found endogenously in chick embryos in the picogram range of concentrations (Wride *et al*, 1994).

Of the two receptors, it has generally been considered that TNFR1 mediates most of the known effects of TNF- $\alpha$  (Heller and Kronke, 1994), including cytotoxicity. TNFR2 has been considered more likely to be involved in the control of cell proliferation, and possibly involved only indirectly in cytotoxicity in co-operation with TNFR1 (Tartaglia and Goeddel, 1992). However, using agonistic antibodies against TNFR1 and TNFR2 on cell cultures, we found no influence on cell proliferation through TNFR2, instead, both receptors appeared to be involved in signalling cytotoxicity (see also Bazzoni *et al*, 1995). Indeed, the effect of TNFR2 in promoting cell death in cell cultures was more pronounced, and its distribution was more striking and localized, than that of TNFR1.

The widespread nuclear localization of TNFR1 in cells from all layers of the embryo both in tissue sections and in cell cultures, may reflect the nuclear translocation of this receptor after ligand engagement and internalization. It is becoming apparent that nuclear translocation of growth factors and their receptors may be a significant means of regulating the transcription of growth factor-responsive genes (Mason, 1994; Prochiantz and Theodore, 1995). The case has thus far been strongest for the nuclear action of fibroblast growth factor, which, after binding at the cell surface, appears to be targeted first to the perinuclear region and then to the nucleus itself. In the early chick embryo (Riese *et al*, 1995) FGF-2 isoforms are found in a predominantly nuclear location in pre-streak stages of development, but are found both in the nucleus and cytoplasm of the epiblast during gastrulation. Although the nuclear functions of FGF isoforms are unknown, it appears likely that there could be direct nuclear effects of these factors on cell proliferation of transcriptional regulation during differentiation. With respect to TNF- $\alpha$  and its receptors, our finding of a nuclear location for TNFR1 complements descriptions in other cells of its abundance in the peri-nuclear zone (Bradley *et al*, 1995), and the

internalization and nuclear translocation of TNF- $\alpha$  itself, paralleling similar translocations of other cytokines such as interleukin-1 (Panagakos and Kumar, 1994). As in the case of FGF, the nuclear functions of TNF- $\alpha$  are as yet unknown.

We observed that the *bcl-2* gene product appears to have a widespread distribution during gastrulation stages of development. This gene, and the related *bcl-x*, which are able to regulate cell survival by blocking pathways to programmed cell death (Reed, 1994; Hockenbery, 1995), are found to be expressed in a number of developmental situations in which cell death is a known contributor to morphogenesis (Eguchi *et al*, 1992; González-García *et al*, 1994; Novack and Korsmeyer, 1994). Further, in some situations, *bcl-2* may be able to prevent TNF- $\alpha$ -mediated cell death (Itoh *et al*, 1993; Vandenabeele *et al*, 1995) and in the present context might provide a means of controlling the action of TNF- $\alpha$ , which is both widely distributed and potentially damaging to the embryo. Similarly, the *Bax* gene is found widely within the embryo, and it also is implicated in the control of cell death by dimerizing with, and thereby regulating the product of, *bcl-2* (Korsmeyer *et al*, 1993). Although the protease, interleukin-1 $\beta$ -converting enzyme (ICE) has been identified with TNF- $\alpha$ -induced pathways to programmed cell death (Miura *et al*, 1995; Nicolson *et al*, 1995; Boudreau *et al*, 1995), we were unable to find its expression in gastrulating embryos. Recent work has shown, however, that ICE is but one member of an expanding family of cysteine proteases involved in cell death processes, so that the cell death described here may not require presence of ICE itself. These ICE-like proteases include, CPP-32, Mch2 and Mch3 (Orth *et al*, 1996), and we have found CPP-32 in avian embryonic tissue at later stages of development (unpublished), indicating its possible involvement.

The observation that TNF- $\alpha$  is effective in inducing DNA fragmentation in embryo culture but not in *in vitro* cell culture, suggests that cytotoxicity *in vivo* may be a synergistic effect involving cytokines working in concert with TNF- $\alpha$ . One such possibility is an interaction between TNF- $\alpha$  and interferon- $\gamma$  (IFN- $\gamma$ ), which has been shown to be synergistic in the promotion of cytotoxicity in several other cell types (Jo *et al*, 1995; Kalovidouris and Plotkin, 1995). The availability of IFN- $\gamma$  during gastrulation has been demonstrated (Rothstein *et al*, 1992).

Our results provide support for the concept that cell death may be controlled in part by integrin-mediated signalling (Ruoslahti and Reed, 1994), and that the perturbation of cell adhesion can result in an apoptotic response (Bates *et al*, 1994; Boudreau *et al*, 1995; Hermiston and Gordon, 1995). We were able to increase levels of TUNEL-positive cells in cultures of endoderm cells, by the addition of anti- $\beta_1$  integrin antibodies to the medium. It is probable that such an effect is responsible for the high levels of cell death observed locally in some regions of the primitive streak (Sanders *et al*, 1996), where changes in integrin engagement accompany the epithelial-to-mesenchymal transformation that occurs as cells traverse the primitive streak and change their primary substratum from laminin and collagen IV to fibronectin and hyaluronan (Sanders, 1986).



Further, it is likely that an adhesion-mediated effect on cell death is responsible for the increased levels of TUNEL-positive cells observed here in cell cultures, but not in embryo cultures, upon application of anti-TNF- $\alpha$  antibodies. We found (Table 2) that both monoclonal and polyclonal antibodies to TNF- $\alpha$  elicited this response to varying degrees. The relationship between TNF- $\alpha$  and cell adhesion is clear from reports indicating that this cytokine is capable of regulating integrin expression (Santala and Heino, 1991; DeFilippi *et al*, 1992), and can interact with extracellular matrix molecules including fibronectin (Alon *et al*, 1994; Coito *et al*, 1995) and laminin (Alon *et al*, 1994; Hershkoviz *et al*, 1995). These effects of TNF- $\alpha$  can result in modulation of cell adhesion to various matrix substrata, and may explain the tendency of anti-TNF- $\alpha$  antibodies to cause detachment of these embryonic cells from fibronectin and the consequent increase in DNA fragmentation. Also in this connection, we have shown an association between the distribution of TNF- $\alpha$  and TNFR2 and the cytoskeleton, suggesting that these antibodies may perturb adhesiveness through an effect on actin microfilaments. Again, there is evidence for interactions between TNF- $\alpha$  and the actin cytoskeleton, with consequent effects on cell adhesion in a number of other cell types (Kohno *et al*, 1993; Deli *et al*, 1995; Tabibzadeh *et al*, 1995).

In summary, we have provided evidence for a potential role for TNF- $\alpha$  and its receptors in the early differentiation of the chick embryo. The pleiotropic effects of this cytokine, which is expressed at various times during development, suggest that it may modulate programmed cell death in the embryo, either by activating cell death signalling pathways directly, or indirectly through perturbation of integrin-mediated cell adhesion and the interaction of cells with their extracellular matrices.

## Materials and Methods

Gastrulating White Leghorn chick embryos, at stage 4–5 of Hamburger and Hamilton (1951), were freed from their yolk and handled in Pannett and Compton's saline or in Tyrode's saline. For whole embryo culture, embryos were explanted according to the method of New (1955), using a 25 mm internal diameter glass ring for support. Embryos were cleaned of yolk using saline washes, and the test solutions (10  $\mu$ l) were added to the exposed surface of the endoderm. The whole embryo cultures were incubated for the requisite length of time at 37°C in a humidified CO<sub>2</sub> incubator.

For cell culture, embryos were dissected using fine tungsten needles, without the aid of enzymatic digestion, to give tissues from the epiblast, mesoderm and endoderm layers. These tissues were explanted in medium 199 with antibiotics onto fibronectin-coated glass coverslips contained in 35 mm diameter plastic petri dishes. The culture medium was supplemented with either 1% fetal bovine serum or with ITS+<sup>TM</sup> (Insulin, Transferrin and Selenium; Becton Dickinson Labware) at a concentration of 10  $\mu$ l stock solution per 1 ml of medium 199. Cultures were incubated for the requisite length of time at 37°C in a humidified CO<sub>2</sub> incubator.

Embryos were prepared for sectioning by fixing with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) overnight at 4°C, washing in PBS (pH 7.4), dehydrating through a graded series of ethanol, clearing in Hemo-De (Fisher Scientific) and embedding in paraffin wax. Blocks were sectioned at 8  $\mu$  thickness.

## The TUNEL technique

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 Wijnsman *et al* (1993). Sections were cleared in Hemo-De (2  $\times$  5 mins), rehydrated through a graded series of alcohol, and were immersed in double distilled water (DDW). Sections were then incubated for 20 min in 2  $\times$  SSC buffer (0.3 M sodium chloride, 30 mM sodium citrate, pH 7) at 60°C, washed in DDW, and incubated in proteinase K buffer (10 mM Tris-HCl, pH 8) for 10 min at room temperature. This was followed by incubation with 2.5  $\mu$ g/ml of freshly prepared proteinase K (Sigma) in 10 mM Tris/HCl, pH 8.0, for 7.5 min at room temperature, and a final wash in DDW. Endogenous peroxidase was inactivated by incubating the sections with 2% hydrogen peroxide in DDW, with 0.5% Tween 20 for 10 min at room temperature. This was followed by a wash in DDW for 5 min. Sections were then incubated in TdT 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, using components from the terminal transferase kit supplied by Boehringer-Mannheim, allowing approximately 80  $\mu$ l for each slide: DDW, 65.6  $\mu$ l; TdT buffer, 5.2  $\mu$ l; cobalt chloride, 2.6  $\mu$ l; biotin-16-dUTP stock (1 nmol/ $\mu$ l), 1.48  $\mu$ l; dUTP, 4.4  $\mu$ l; and TdT (10 units/ $\mu$ l), 1.6  $\mu$ l. The sections were exposed to reaction mixture and incubated at 37°C for 1.5 h in a humid chamber. The reaction was terminated by immersion of the slides in 2  $\times$  SSC at room temperature for 15 min. Following two further washes in PBS (pH 7.4), the sections were covered in 3% skimmed milk (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<sup>®</sup> (Sigma), diluted 1:50 in 3% SM in PBS with 0.5% 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 dimethyl formamide 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  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub> was then added. This solution was filtered and applied to the sections which were monitored under a microscope until the color reaction was optimal. The slides were then washed in PBS and mounted in Crystal Mount<sup>®</sup> (Fisher Scientific Inc.). Positively stained nuclei were colored intensely red.

In addition, both positive and negative controls were performed. In the negative controls either the bio-16-dUTP stock or the TdT enzyme was omitted from the reaction mixture. For positive controls, sections were treated with DNaseI buffer (30 mM Trizma base, 140 mM sodium cacodylate, 4 mM magnesium chloride, 0.1 mM dithiothrei-

tol) for 5 min following the quenching of endogenous peroxidase, incubated with fresh DNaseI (1  $\mu$ g/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 DNaseI, every nucleus was stained intensely red.

For cultures, the TUNEL method was carried out as above. Fixed cultures were washed with PBS and the procedure was started at the hydrogen peroxide step to inactivate endogenous peroxidase, having *not* been treated with proteinase K.

### Sources of reagents

Recombinant murine TNF- $\alpha$  was obtained from Genzyme Corp. Antibodies against murine TNF- $\alpha$  were obtained from: Genzyme Corp. (polyclonal); Upstate Biotechnology Inc., (monoclonal, clone MP8 XT22); abV ImmuneResponse Inc., Endogen Inc., and Gibco-BRL (monoclonal, clone MP6 XT22). Agonistic polyclonal antibodies (Tartaglia *et al*, 1991) against murine TNFR1 and TNFR2 were obtained from Genentech Inc. and Santa Cruz Biotechnology Inc. Bromodeoxyuridine (BrdU) was obtained from Sigma and a monoclonal antibody against BrdU was obtained from Amersham Life Science Inc. Monoclonal antibody to chicken integrin  $\beta_1$  complex, (V<sub>2</sub>E<sub>9</sub>), was obtained from the Developmental Studies Hybridoma Bank. Polyclonal antibodies to amino acid sequences from *bcl-2* (#SC-492), *Bax* (#SC-493) and *ICE* (#SC-514), together with their control peptides, were obtained from Santa Cruz Biotechnology Inc.

### Immunocytochemistry

Sections were de-waxed, rehydrated, and treated with 1% or 4% bovine serum albumin (BSA) in PBS to block non-specific staining. Primary antibodies were applied at dilutions of between 1:50 and 1:200 in PBS plus BSA for 1–2 h. For immunolabelling with antibodies to *bcl-2*, *Bax* and *ICE*, antibodies were applied at a dilution of 1:100 at 4°C overnight. After washing several times with PBS plus BSA an appropriate second antibody, labelled with fluorescein isothiocyanate, was applied at a dilution of 1:100 for 1 h. Slides were finally washed with PBS and mounted.

Cultures were fixed overnight with 4% formaldehyde, and then washed with PBS plus BSA containing 0.5% Tween 20 to permeabilize the cells. Cultures were then treated as above.

In control slides, the primary antibody was replaced by PBS and either rabbit serum, for polyclonal antibodies, or mouse IgG for monoclonal antibodies. In the case of *bcl-2*, *Bax* and *ICE* antibodies, a pre-absorption control was carried out using the peptide provided with the antibody (peptide:antibody::1:1, final antibody concentration 1:100). In all cases, the control slides showed very low levels of non-specific background staining which was clearly distinguishable from the staining of the experimental slides.

### Western blotting

Polyacrylamide gel electrophoresis was carried out as described previously (Wride and Sanders, 1993), except that embryos were homogenized in a buffer containing protease inhibitors as described by Jaskoll *et al* (1994). Western blotting was also carried out as described previously, except that bands were detected using an enzyme chemiluminescence kit obtained from Amersham Life Science Inc.

### Mouse cell culture

Mouse embryo at the early- to mid-primitive streak stage (Downs and Davies, 1993) were obtained from timed-pregnant females. Embryos were finely minced with tungsten needles, and the pieces explanted into 199 culture medium with antibiotics and 1% fetal bovine serum. Cultures were incubated as described above. No attempt was made to separate embryonic germ layers in these cultures.

### Bromodeoxyuridine (BrdU) incorporation

Cell cultures were grown overnight on fibronectin-coated coverslips as described above. Next day, the medium was replaced with medium containing 10  $\mu$ g/ml BrdU, and the cultures were incubated for a further 2 h before rising with Tyrode's saline and fixing overnight. After rinsing with PBS, cultures were treated with 1% hydrogen peroxide in 50% methanol for 30 min, followed by another wash with PBS. Following treatment with 1% BSA and 0.1% Tween 20 in PBS for 30 min, cultures were incubated with monoclonal antibody to BrdU, undiluted, for 1 h. Controls were incubated with PBS. Cultures were washed with 1% BSA, incubated with biotinylated goat anti-mouse IgG, washed again, and treated with Extra-Avidin peroxidase (Sigma), diluted 1:50 in PBS containing 4% BSA, for 30 min. Colour was developed using the AEC procedure as described above.

### Quantification of TUNEL and BrdU labelling

Cultures, with cell nuclei labelled using either the TUNEL or BrdU incorporation procedures, were quantified using the 'Image-1' image analysis system (Universal Imaging Corp, West Chester, PA), and a Zeiss Axioskop microscope. The image was captured and thresholded to highlight only structures stained to an appropriate density, and the 'shape factor' was adjusted so that only labelled nuclei were counted as a function of culture area. Results were expressed as the number of labelled nuclei per standard, but arbitrary, area of spread cells, and were analyzed for statistical significance using an unpaired t-test.

Analysis of the incidence of nuclei undergoing DNA fragmentation in sections of embryos that had been treated while in culture by the method of New (1955), was carried out as above by expressing the number of TUNEL-positive nuclei per area of tissue in sections of standard thickness (5  $\mu$ m). These sections were always taken from the same level of the embryo – midway along the primitive streak – and always encompassed the same section width,

including the entire primitive streak and a 60  $\mu\text{m}$  stretch of tissue to one side of the streak, similar to the region shown in Figure 5b.

## Acknowledgements

We thank the Medical Research Council of Canada for an operating grant in support of this work, and the Alberta Heritage Foundation for Medical Research for a studentship to MAW. We also thank the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, and by the Department of Biological Sciences, University of Iowa, Iowa City, under contract from the NICHD.

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