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PROGRAMMED CELL DEATH IN POST-TRAUMATIC BONE CALLUS

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Abstract - Some osteoblasts in the expanded population of periosteal cells that occurs following bone injury are removed from the callus by apoptosis. Our objective was to study whether the consequences of activation of the death program could include feedback control of the healing response. Transforming growth factor ß and interleukin-1ß were delivered together continuously to a standardized tibia] defect in rats for 3 days using implanted micro-osmotic pumps. The bones were recovered at 1. 2. 3, 5, 7, 10 and 14 days after injury (n=6 in each treated and control group) and concentrations of proliferating cells. osteoblasts and apoptotic bodies were determined. The injury-induced apoptotic component of the healing response was shifted in time due to the combined cytokines. compared with vehicle only. with the result that the peak in the concentration of apoptotic bodies occurred 2-3 days earlier in the treated animals. Neither osteoprogenitor proliferation nor osteoblast concentration was affected by addition of the cytokines. The results suggested that activation of apoptosis during injury repair was not necessarily a passive consequence of the cellular response to injury. Programmed cell death could therefore have an active role in modulating hone repair.

Key words: Programmed cell death, apoptosis, interleukin1ß, transforming growth factor ß, growth regulation, osteoblast

Abbreviations: IL-1: interleukin-1; **TGFß**: transforming growth factor beta; **ABs**: apoptotic bodies: **TUNEL:** transferase-mediated biotin-dUTP nick end-labelling

INTRODUCTION

Programmed cell death (apoptosis) is a generally utilized mechanism for disposing of an excess population of cells via implementation of a genetic program, and the excess cells associated with bone are no exception. Apoptosis results in the removal of cartilage cells at the growth plate (Gibson, 1998; Lee *et al*., 1998), and in elimination of osteocytes following completion of modeling during development (Bronckers *et al*., 1996; Lynch *et al*., 1998; Rice *et al.,* 1999). In remodeling, both the osteoclasts responsible for sculpting bone and the osteoblasts that subsequently synthesize new bone were reported eliminated by activation of death programs (Hughes and Boyce, 1997; Jilka *et al*., 1998). Previously, we have developed an animal model to study the bone injury-response system present in the periosteum (Landry *et al.,* 1996), and discovered that apoptosis was a routine feature of the osteogenesis occurring in the model. Following bone injury, apoptosis was seen in the tissue compartment containing the osteoblasts and osteoprogenitor cells, and was coordinately regulated with osteoblast levels (Landry *et al*., 1997). Ultrastructural evidence established that osteoblasts underwent apoptosis and were phagocytosed by nearby intact osteoblasts (Olmedo *et al.,* 1999).

We studied the question of how the time-dependent pattern of injury-induced proliferation and differentiation in the periosteum was regulated, and showed that interleukin-1ß (IL-1ß) increased the amount of osteoblast formation induced by injury (Olmedo *et al.,* 1999). Because IL-1ß did not affect proliferation, we suggested that the increased osteoblast levels resulted from increased differentiation. The number of apoptotic bodies per osteoblast was also affected by the exogenous cytokine, but how this was related to the change in osteoblast concentration was not determined.

Transforming growth factor ß (TGFß), a potent osteotropic cytokine (Bolander, 1992; Sandberg *et al*.. 1993). inhibited osteoblast differentiation in bone nodules (Harris *et al.,* 1994) and antagonized the effects of IL-1 in cell systems (Chandrasekhar and Harvey, 1988; Andrews *et al.;* 1989) and *in vivo* (van Beuningen *et al*., 1994). TGFß induced apoptosis in several human and animal cell lines (Chen and Chang, 1997; Ohta *et al*., 1997; Brown *et* al., 1999; Guo and Kyprianou, 1999).

We reasoned that if TGFß antagonized the effects of IL-1ß on osteoblast concentration in our model (Landrv *et al.,* 1997), that result would further support a regulatory role for IL-1ß in the bone-injury response and might clarify the role of programmed death in shaping the response. Accordingly, the objectives of this study were to determine the effect of TGFß on the IL-1ß-induced increased rate of osteoblast differentiation, and to assess whether regulation of cell death was altered during the healing process as a consequence of the cytokine treatment.

MATERIALS AND METHODS

A bone defect, 1.1 mm in diameter and 0.5 mm deep, was made unilaterally in the tibia of male Fischer rats (200-250 g) (Landry et al., 1996). The defect did not communicate with the medullary canal, thus eliminating the influence of the intramedullary bone-repair system and isolating for study the system present in the cambial periosteum. The operative site was irrigated to remove hone particles that could initiate bone induction, and micro-osmotic pumps (Model 1003D, Alzet. Palo Alto, CA) loaded with either TGFß and IL-1ß (Promega, Madison, WI) dissolved in phosphate-buffered saline containing 1 mg/ml bovine serum albumin, or vehicle only, were implanted subcutaneously in the upper limb. The pump output tubing (polyvinyl chloride, 0.64 mm OD (Bolab, Lake Havasu City, AZ) was sutured to the soft tissue outside the region of interest, and the discharge port was positioned directly over the hone defect. The skin was closed with sutures. and the rats were caged individua11 and fed and watered *ad libitum*. Each pump delivered 3.7 ng TGFß and 0.5 ng IL-1ß per microliter per hr. for 72 hrs. or, in the controls, an equivalent volume of vehicle. The concentrations were chosen on the basis of in vitro reports suggesting that 0.1-10.0 ng/ml was associated with altered protein and DNA synthesis by bone cells (Gowen *et* al., 1985; Smith *et al*., 1987; Harris *et al*., 1994). Cytokines were delivered for 72 hrs. following injury because that is the period during which they are maximally expressed in fracture callus (Einhorn *et al*., 1995).

The bones were recovered at 1, 2, 3, 5, 7, 10 and 14 days post-injury (n=6 in each treated and control group). Earlier work showed that these recovery times were necessary and sufficient to permit a reliable determination of the time-dependent pattern

of cellular changes that occurred following injury. In a separate study, to evaluate the effect of TGFß alone on the bone injury response, implanted pumps delivered 3.67 or 36.7 ng TGFß per microliter per hour for 2 hrs. or, in the controls, an equal volume of vehicle, after which the animals were sacrificed.

One hour prior to sacrifice (carbon dioxide suffocation), the animals that received both cytokines were pulse-labelled with tritiated thymidine (intraperitoneal injection, 1 μCi/gram of body weight, ICN Biomedicals, Costa Mesa, CA). All animal procedures were carried out between 10:00 am. and 2:00 p.m. to minimize potential effects due to circadian rhythms, and the procedures were approved by the Institutional Animal Care and Use Committee.

The specimens were fixed in 10% neutral buffered formalin and decalcified (Ca-EX, Fisher Scientific, Pittsburgh, PA). A 10-mm segment of bone centered on the bone defect was embedded in epoxy (JB-4, Polysciences, Warrington, PA) and completely sectioned in the longitudinal plane, and 3 trios of sections were selected from the lateral, central and medial aspects of the defect. One section from each trio was dipped in photographic emulsion (Ilford K5, Polysciences, Warrington, PA), processed for radioautograph and counterstained with van Gieson's: a second set of sections from each trio was stained with methyl-green/thionin, and the third set with toluidine-blue/basic-fuchsin.

Osteoblasts were evaluated in the sections stained with methyl-green/thionin. A cell was identified as an osteoblast if it was located in the cambial compartment [previously shown to contain only alkaline-positive cells (Landry *et al.*, l996)], was approximately 15 μm in its longest dimension, contained a nucleus with nucleoli, and exhibited extensive basophilic cytoplasm with a prominent Golgi apparatus. Proliferation was assessed in the radioautographs by counting the number of cells that had more than 5 grains per nucleus. Quantitative determinations of apoptotic bodies (ABs) were made in the sections stained with toluidine-blue/basic fuchsin. ABs were identified as membrane-bound structures containing various ratios of pyknotic chromatin and condensed cytoplasm (Kerr *et al.*, 1972; Ferguson and Anderson, 1981; Kerr *et al.*, l987). They ranged in size from 1-7 μm and were distinguished from lymphocytes and mitotic figures on the basis of size and in the presence of a cytoplasmic halo, respectively (Montironi *et al.*, 1994). All intra- and intercellular structures that met these criteria were counted as ABs.

Separate counts for osteoblasts, proliferation, and ABs were made in the cambium within ± 3 mm from the center of the defect, and within the defect, and each count was normalized by its corresponding length along the bone axis. All counts were made in a blinded fashion and were reproducible to better than 10%. The results were averaged over the three sections, and the grand means of all the animals that received a given treatment and were recovered at a specific time after injury were used to estimate the continuous time-dependent functional changes of the cellular parameters. A Mathcad program (MathSoft, Cambridge. MA) was developed to ascertain the least-squares fit of the data to the generalized function $y(t) = a + b \cdot t + c \cdot t^2 \cdot e^{-x} + d \cdot t^2$. The input to the program was the grand mean of each parameter at each time, t, and the output was a set of coefficients (a, b, c, d). Substitution of the coefficients into the generalized function resulted in the particular function that was the least-squares fit to the data (Burden and Faires, 1997) and was used for illustrative purposes. Time of peak response was determined as the time corresponding to the maximum value of the respective computed function. Other generalized functions were also employed to generate a range of curves for analysis of peak response using the t test at $p<0.05$.

RESULTS

Pertinent characteristics of the model system were described previously (Landry *et al.*, 1996). Briefly, the resting cambium consisted of 2-5 layers of alkaline phosphatase-positive cells that had a spindle-shaped darkly-staining nucleus and scanty resolvable cytoplasm at 400x. The cells were morphologically identical, but were distinguishable on the basis of location and response to injury. The bone-lining cells were located on the cortical surface and activated following injury to become osteoblasts. The osteoprogenitor cells were located above the bone surface. They also activated after injury, from which state they either divided or differentiated to become osteoblasts. Bone matrix formed on the uninjured cortical surface, and mineralization commenced 2-3 days after injury. Callus first appeared in the defect by centripetal growth from the periphery beginning around day 5. Thus, an adequate description of the time-dependent changes that occurred in the model during the first 2 weeks of healing required separate characterizations of the cells next to and in the defect.

The injury produced the expected strong increase in osteoblast levels above baseline, but the time dependence of the post-injury response patterns was essentially identical in the cytokine and control groups when assessed in the region next to the defect, or in the defect (Fig. 1). Because IL-1ß alone increased osteoblasts compared with vehicle only (Olmedo *et al.*, 1999), the data in fig. 1 indicated that TGFß antagonized the effect of IL-1ß. The time dependence of proliferation in the control

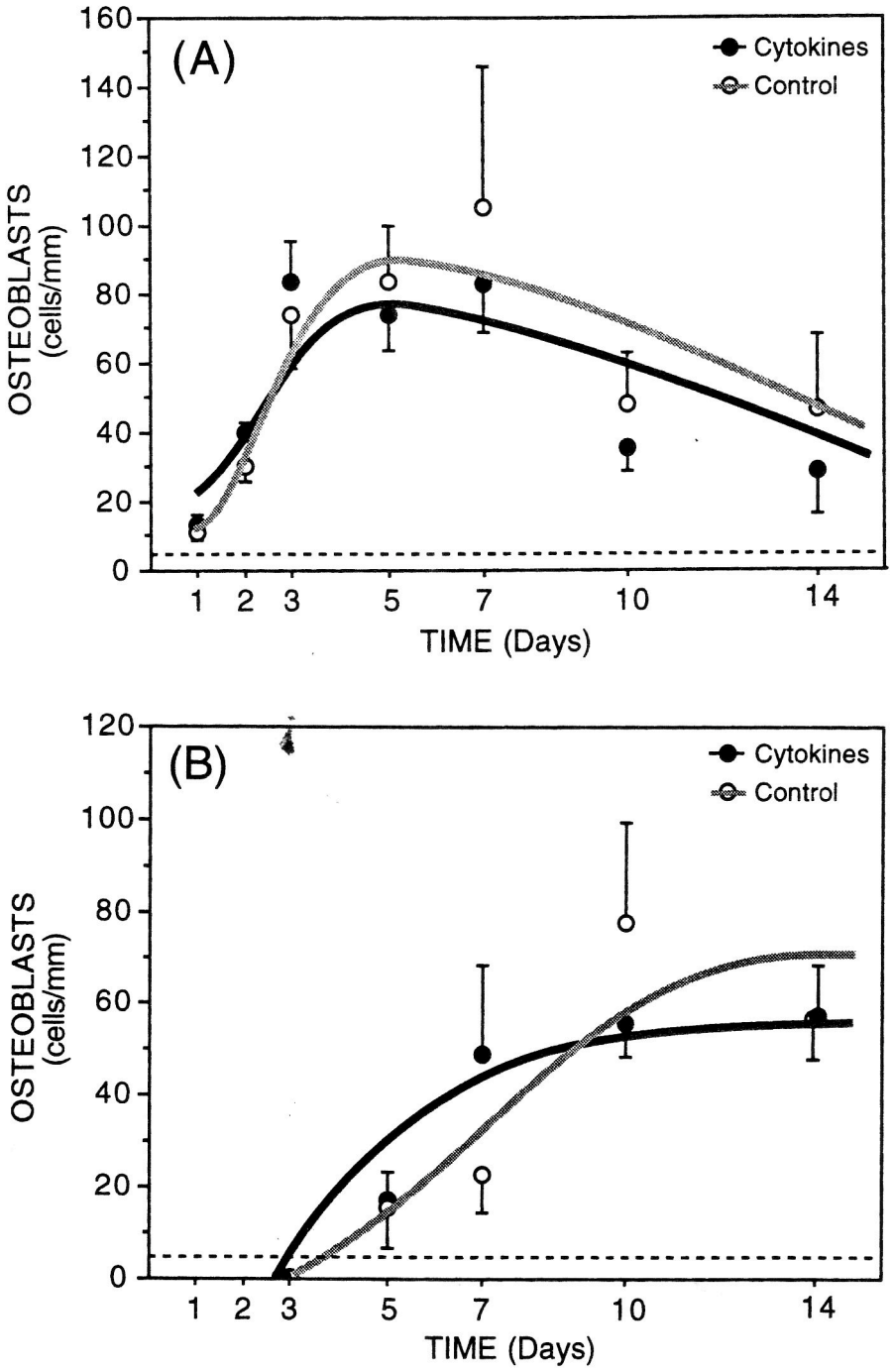

Fig. 1 *Effect of TGFβ and IL-1β on osteoblast concentration (mean ± SE) following bone injury* (**A**) *in the cambium of the uninjured bone,* ± 3 mm of the defect center, and (**B**) *in the defect.* The dashed line indicates osteoblast concentration in uninjured bone.

rats induced by the injury was essential the same as that described earlier (Landry *et al.*, 1997), and the proliferation was unaffected by the addition of the combined cytokines, both next to and in the defect (Fig. 2). In contrast, the pattern of post-injury apoptosis was altered by addition of the cytokines in both regions of the injury site (Fig. 3). The peak in the concentration of ABs following injury occurred earlier in the rats that received the cytokines, compared with the controls (Table 1).

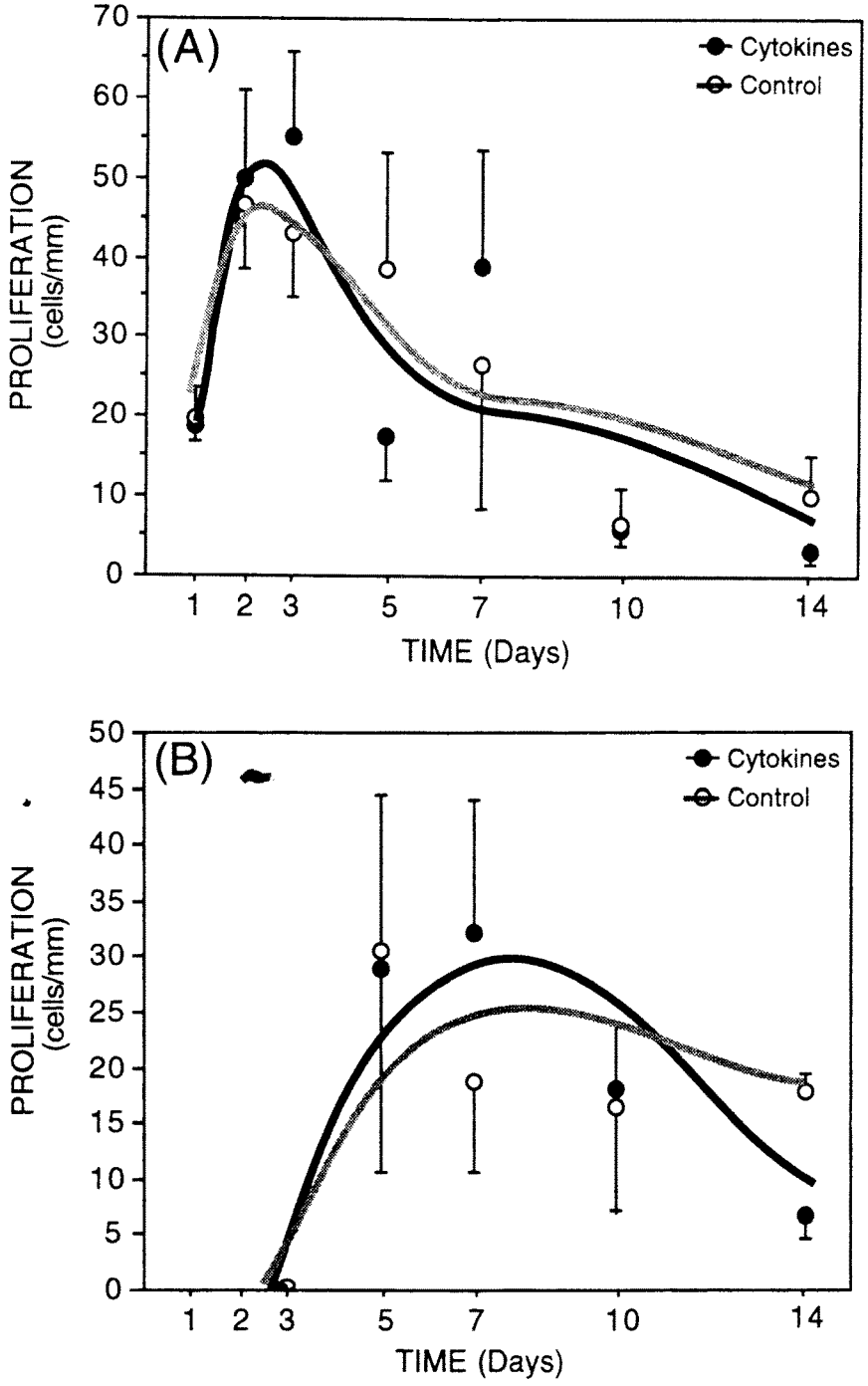

Fig. 2 *Effect of TGFβ and IL-1β on proliferation* (mean ± SE) *following bone injury* **(A)** in *the cambium of the uninjured bone, ± 3 mm of the defect center, and* **(B)** *in the defect*. The proliferation in uninjured bone was <1 cell/mm

In rats that received TGFß alone, the osteoblast concentration in the cambium adjacent to the defect at 3 days after injury was 48.0 ± 9.6 cells/mm (N=7. mean ± SE), compared with 51.0 ± 3.7 cells/mm in the animals that received vehicle only (N=l0). Thus addition of TGFß alone had no effect on osteoblast concentration.

Table 1 *Number of days after injury at which the maximum response occurred in cytokine-treated and control rats, as determined from the least-square curves.*

	Days after injury until maximum response					
	Osteoblast Concentration		Proliferation		Apoptosis	
	Cambium	Defect	Cambium	Defect	Cambium	Defect
IL-1ß/TGFß	5.1	14.2	2.2	7.5	5.1*	7.5*
Control	5.7	15.9	2.3	7.1	7.1	10.9

*p<0.05

DISCUSSION

We addressed the question of the type of role played by apoptosis in the bone-healing response. Bone injury resulted in formation of a complex local environment containing time-dependent levels of regulating mediators whose interactions controlled cell activation, proliferation, differentiation, protein expression, matrix mineralization and modeling. One possibility was that the consequences of injury-induced apoptosis were neutral in the sense that, following commitment to and execution of the death program, phagocytosis of the cell fragments could have occurred by nearby cells in the absence of any local cellular metabolic changes that affected the speed of healing. Alternatively, apoptosis might have had a more active role, perhaps as part of a feedback system in which early apoptotic events regulated local release of agents that modulated healing. For example, feedback control might have opposed the inflammation that originally elicited the cellular response by down-regulating IL-1ß and up-regulating TGFß (Fadok *et al.,* 1998). In distinction to necrosis, apoptosis does not trigger an inflammatory response. This fact has encouraged an emphasis of the occurrence and mechanism of programmed cell death at the expense of its possible consequence to the remaining cells (Gibson, 1998). Nevertheless, there is evidence for an active role of apoptosis in monocytes (Voll *et al.,* 1997), in macrophages (Fadok *et al.*. 1998) and in chondrocytes (Gibson, 1998), and such might be the case in bone healing. We approached the question experimentally by asking whether it was possible to specifically alter the pattern of apoptosis that occurred following bone injury. TGFß alone had no effect on osteoblast levels, but when it was added simultaneously with IL-1ß, the previously established effect of IL-1ß on osteoblast concentration (Olmedo *et al.,* 1999) was opposed (Fig. 1). As was the case with IL-1ß alone (Olmedo *et al.,* 1999), endogenous regulation of proliferation at the injury site was unaffected by introduction of the combined cytokines (Fig. 2). However, introduction of both agents together altered the timing of the execution of the death program of bone cells (Fig. 3). The post-injury peak in the number of apoptotic bodies present in the cambium adjacent to the defect occurred 2 days earlier in the cytokine-treated animals, and in the defect the peak occurred more than 3 days earlier (Table 1). Thus, the novel result was that the environment at the site of healing could be manipulated so that apoptosis was altered, but not proliferation or differentiation (as reflected in osteoblast levels). Our ability to specifically alter the time of execution of the death program in bone cells following injury suggested that apoptosis is not necessarily a passive consequence of the proliferation and osteoblast formation elicited by the injury. One possibility is that the cellular changes and apoptosis are independently regulated, but a more likely possibility is that they can be coupled via feedback. For example, phagocytosis of ABs by osteoblasts could inhibit their production of IL-1ß and increase their production of TGF which might

then trigger more bone cells to execute their death program. Although it is possible that apoptotic changes could feed back at one or more levels of osteogenesis. whether or not this actually occurs, either in the model or more generally, and how the feedback might be accomplished are issues that were not addressed in this study.

Inter-animal variation in healing was a major limitation in the model, but a fundamental one, and it

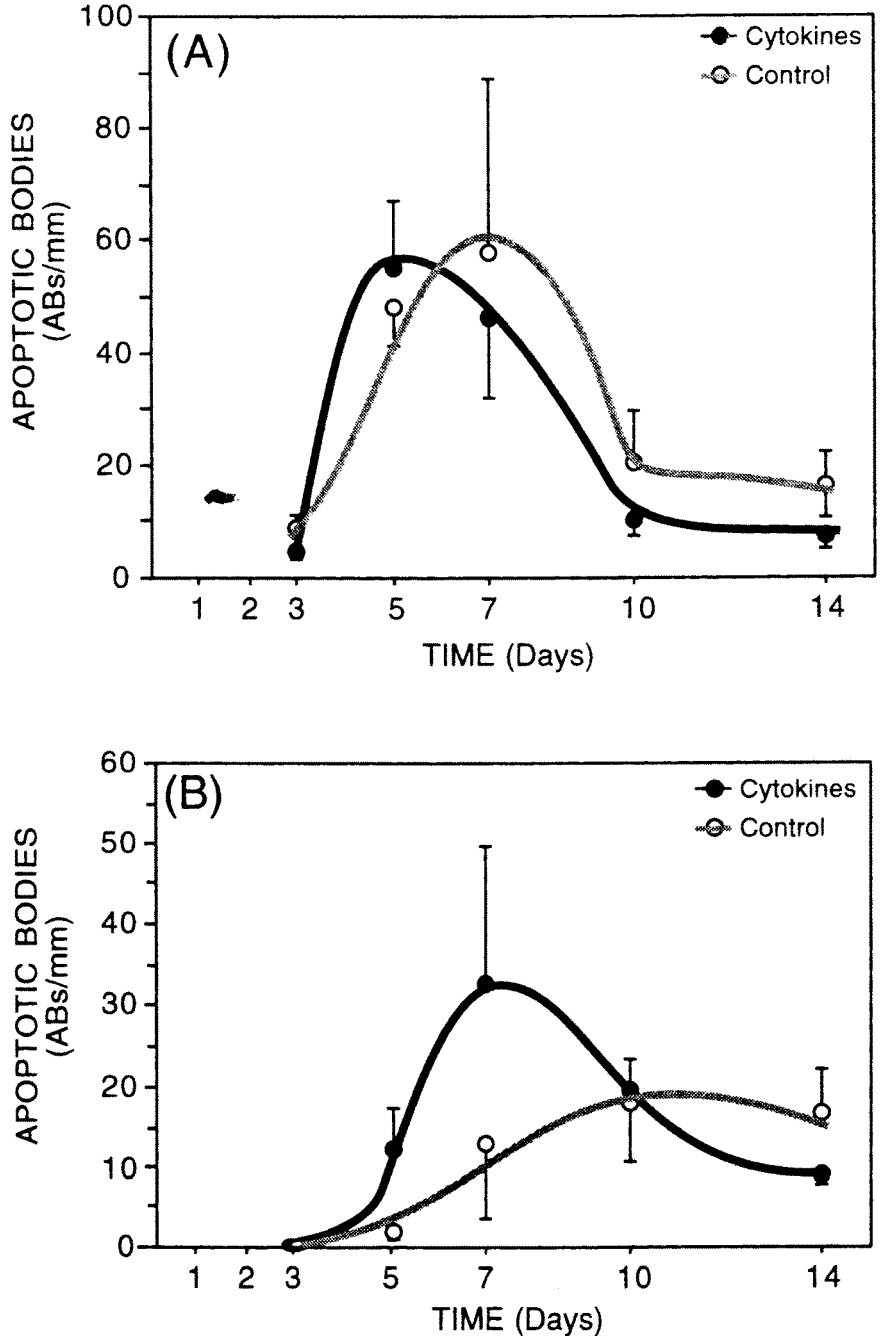

Fig. 3 *Effect of TGF,8 and IL-] on concentration of apoptotic bodies* (mean ± SE) *following bone injury* **(A)** *in the cambium of the uninjured bone, ± 3 mm of the defect center, and* **(B)** *in the defect.* The concentration of apoptotic bodies in uninjured bone was <1 AB/mm.

inevitably shaped the kinds of hypotheses that could be tested. We realized what we think to be a near-maximum attempt to control variability in the bone response to injury All animals were operated on by the same experienced surgeon. The defect was limited to the periosteum and designed so that cartilage was not a component of the healing response. All animals were operated on and recovered within relatively narrow time windows to minimize circadian variability. The defect site was sectioned in its entirety so that the sections selected for analysis came from known locations and were truly representative. Individual counts based on morphological criteria were reproducible to better than l0. Despite that, during these steps large variations were observed, particularly at days 5-10 see Figs. 1-3. The strategy we followed was to evaluate the global healing pattern as manifested in each parameter because the global pattern was less responsive to individual variation. Proliferation. for example. which was not affected by cytokine treatment. yielded patterns of time dependence that were essentially identical in different groups (Fig. 2) and different studies (Olmedo *et al*., 1999).

It is worthwhile to consider some of the advantages and disadvantages of cellular versus animal models in relation to study purpose. Information can be gained regarding the mechanics of the death program by parsing individual steps in various cell lines (Chen and Chang, 1997; Brown *et al.,* 1999). The results of such studies generally suggest that it is executed in all cells by more or less the same set of proteolytic elements and substrates (Thornberrv and Lazebnik, 1998). But the metabolism of pure populations of cells is artifactual because they lack the complex environmental feedback normally experienced in tissues. For example, osteoblast-like cells readily divide in culture. whereas osteoblasts. which are the preponderant number of excess cells that must he removed in callus, do not divide. *In vitro* studies, therefore, are able of providing information only concerning what can occur. and the question to know the type of processes occurring during any form of osteogenesis remains unresolved. On the other hand, use of the Transferase-mediated, biotin-dUTP nick end-labelling (TUNEL) technique for demonstrating apoptosis based on DNA laddering is of limited use in tissue because it is difficult to recover cells from callus, and because TUNEL is susceptible to both positive and negative artifacts (Gibson, l998). There is, presently, no more reliable method for quantitating bone-cell apoptosis in tissues than the tedious method of actually counting the number of apoptotic bodies.

Animal models are directly relevant to the issue of identification of the factors that actually cause cells to invoke their death program, particularly in the context of bone healing. Apoptosis in bone callus is ultimately a result of a trauma or of other specific external stimuli, and thus somehow must be proportionately regulated and influenced by the external stimuli. This contrasts with the causes of apoptosis in other forms of osteogenesis where the genetic mechanism of programmed cell death is itself subjected to genetic control (Bronckers *et al.,* 1996; Hughes and Boyce, 1997; Gibson, 1998; Lynch *et al*., 1998). In these cases there is a diffuse relation, if any, between apoptosis and external factors.

Further efforts aimed at extricating the complex regulatory pathways that regulate bone healing are needed to understand how programmed cell death functions as a regulatory element in the system governing injury-induced bone growth and, more generally, how injured bone normally exhibits a stable and predictable response.

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