

Impaired Intramembranous Bone Formation during Bone Repair in the Absence of Tumor Necrosis Factor-Alpha Signaling

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Key Words

Tumor necrosis factor-alpha · Fracture repair · Bone repair · Chondrogenesis · Osteogenesis

Abstract

Tumor necrosis factor-alpha (TNF- α) is known to mediate bone resorption; however, its role in osteogenesis has not been fully elucidated. In order to investigate the direct role of TNF- α signaling in the recruitment and differentiation of osteoblasts, two separate models of bone repair were used, marrow ablation and simple transverse fractures. These models were carried out in the tibiae of both wild-type and knock-out mice in which both TNF- α receptors (p55^{-/-}/p75^{-/-}) had been ablated. Marrow ablation is a unique model in which robust intramembranous bone formation is induced without an endochondral component, followed by remodeling and restoration of the original trabecular architecture of the bone marrow. In contrast, fracture repair proceeds concurrently through both endochondral and intramembranous processes of new bone tissue formation. In both models of bone repair, healing was delayed in the TNF- α receptor (p55^{-/-}/p75^{-/-}) deficient mice. In the marrow ablation

Abbreviations used in this paper

AP1	activating protein one
BMP	bone morphogenetic protein
Cbfa 1	core binding factor a-one
cRNA	complementary RNA
DMEM	Dulbecco's modified Eagle medium
Fas	a type of ligand; a transmembrane protein of the TNF receptor family
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
L32	a type of mRNA
LT	lymphotoxin
LT- α	lymphotoxin-alpha (aka TNF- β)
LT- β	lymphotoxin-beta
mRNA	messenger RNA
NF κ B	nuclear factor-kappa b
OPG	osteoprotegerin
OPG-L	osteoprotegerin ligand (aka RANK-L or TRANCE)
RNase	ribonuclease
RPA	ribonuclease protection assay
Sox 9	Sry (sex-determining region γ)-related HMG (high mobility group) box
TGF- β	transforming growth factors-beta
TNF	tumor necrosis factor (a cytokine family)
TNF- α	tumor necrosis factor-alpha
TNF- β	tumor necrosis factor-beta (aka LT- α)

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1422-6405/01/1693-0285\$17.50/0

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model, young osteoblasts were recruited into the marrow space by day three in the wild-type mice, while the TNF- α (p55^{-/-}/p75^{-/-}) mice had only granulation tissue in the marrow cavity. Type I collagen and osteocalcin mRNA expressions were reduced ~30 and ~50%, respectively, of the control values in the TNF- α receptor ablated mice. In the fracture repair model there was almost a complete absence of the initial intramembranous bone formation on the periosteal surface in the TNF- α (p55^{-/-}/p75^{-/-}) mice. As healing progressed however, the callus tissues were greatly enlarged, and there was a delay in hypertrophy of the chondrocytes and the resorption of cartilage tissue. While during the initial period of fracture repair there was a marked reduction in the expression of both type I collagen and osteocalcin mRNAs in the TNF- α (p55^{-/-}/p75^{-/-}) mice, levels of these mRNAs were elevated by ~10–20% over the wild type at the later time points in the absence of endochondral resorption of the callus. The lack of inhibition of osteogenesis during endochondral resorption suggests that a different set of signals are involved in the recruitment of osteogenic cells during endochondral repair than during intramembranous bone formation. Co-culture of chondrocytes with a mesenchymal stem cell line was carried out to examine if chondrocytes themselves produced paracrine factors that promote osteogenic differentiation. These experiments demonstrated that chondrocytes do indeed produce factors that promoted osteogenic differentiation. In summary, the results presented here suggest that TNF- α plays a crucial role in promoting postnatal bone repair through the induction of osteoprogenitor cell recruitment or osteogenic cell activation in the context of intramembranous bone formation. These results further suggest that the signals that promote osteogenesis during endochondral bone formation are different from those involved in intramembranous bone formation.

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Introduction

The processes of fracture healing and skeletal tissue repair are complex, involving the coordinated participation of multiple cell types including cells of the hematopoietic and immune systems, in conjunction with the mesenchymal cells of the skeletal and vascular systems [Einhorn, 1998; Buckwalter et al., 2000]. Many of the molecular mechanisms known to regulate skeletal cell differentiation during embryological development have also

been investigated during bone repair. These have included studies on the role of the TGF- β /BMP family of morphogens [Barnes et al., 1999], and the molecular mechanisms that control cartilage and bone cell specific gene transcription such as Cbfa 1 [Ducy et al., 1997; Otto et al., 1997] and Sox 9 [Lefebvre et al., 1998; Bell et al., 1997]. Local regulation of skeletal tissue differentiation and metabolism by specific growth factors, cytokines, and morphogens during postnatal tissue repair, however, is probably very different from that of embryological development. Fracture and bone repair after injury occurs in a post-embryological context in close physical association with the bone marrow, is affected by many factors in the peripheral circulation, and takes place in response to an inflammatory process that has been initiated by trauma.

The tumor necrosis factor (TNF) cytokine family has been shown to be essential in the mediation of both the innate inflammatory process [Dinarello and Mier, 1987; Feghali and Wright, 1997] in response to tissue injury, as well as being a crucial regulatory component in bone remodeling [Bertolini et al., 1986; Gowen et al., 1990; Kimble et al., 1997]. Two recent reports also suggest that TNF- α cytokines are involved in the normal mediation of both fracture and bone repair [Shimizu et al., 1998; Hashimoto et al., 1989]. The TNF family of cytokines and their receptors is currently composed of some ten known ligands and receptors. The members of this cytokine family that have been the most extensively characterized are TNF, lymphotoxin (LT) and Fas ligand [Bazzoni et al., 1996; Beutler, 1996; Nagata, 1997]. The TNF family members that are most homologous are TNF- α , TNF- β (LT- α) and LT- β . Both TNF ligands exist as homotrimers, while the LT- β exists only as a heterotrimer of (LT- α)₁(LT- β)₂. There are three receptors in this family, TNFR1 (p55), TNFR2 (p75) and LT- β receptor. Both TNF ligands bind both TNF receptors, but LT- β /TNF- β trimers only bind to the LT- β receptor. The TNF cytokine family has been studied the most extensively for its role in immune function. These molecules are induced in response to both infection and a multitude of inflammatory stimulants. They are shown to mediate two primary responses; either eliciting programmed cell death (apoptosis) or facilitating cell survival and growth through primarily the activation of the NF κ B and AP1 transcription factors. The dichotomy of cellular responses to these cytokines resides in the receptors that are activated and the downstream signal transduction molecules that interact with these receptors [Wallach et al., 1999].

TNF- α has been extensively studied in bone and cartilage metabolism and for a number of years has been

implicated in the mediation of osteoclastogenesis [Bertolini et al., 1986; Gowen et al., 1990; Kimble et al., 1997]. Monocytic levels of TNF production have been shown to be elevated in surgical or natural menopausal states, and a number of studies have shown that TNF- α can induce osteoclast formation in vitro [Kimble et al., 1997]. More recently, both a novel member of the TNF receptor family, osteoprotegerin (OPG), and its specific ligand OPGL have been shown to be key regulators controlling bone mass through their regulation of the resorptive cycle [Simonet et al. 1997; Bucay et al., 1998; Lacey et al., 1998; Takahashi et al., 1999; Hofbauer et al., 2000]. Recent studies clearly indicate that apoptosis of chondrocytes plays an important role in endochondral ossification [Hashimoto et al., 1997; Horton et al., 1998]. Two members of the TNF family of cytokines, Fas ligand and TNF- α , have both been implicated in providing death signals to chondrocytes. Treatment of human articular chondrocytes with Fas ligand in vitro causes apoptosis and the Fas system has been shown to be present in growth plate chondrocytes in vivo, leading to the suggestion that it might play a role in chondrocyte apoptosis during endochondral development [Roach et al., 1996; Gibson et al., 1997; Lee et al., 1998]. In recent studies, our laboratories have shown that TNF- α can induce apoptosis in vitro in populations of growth chondrocytes [Aizawa et al., 2001]. A growing body of data has also shown that TNF- α plays a major role in the pathogenesis and destruction of joints in rheumatoid arthritis, suggesting that it has profound biological effects on normal cartilage metabolism [Lipsky et al., 2000]. The focus of the current studies was to determine the role of TNF- α in the mediation of either endochondral bone repair or intramembranous bone repair.

Materials and Methods

Materials

Histological reagents and chemicals were purchased either from Sigma Chemical Company (St Louis, Mo., USA) or Fisher Scientific (Springfield, N.J., USA). RPA reagents were from PharMingen Corp. (San Diego, Calif., USA). Homozygous knock-out mice in which the genes for both TNF- α receptors (p55^{-/-}/p75^{-/-}) were targeted [Peschon et al., 1998] and strain matched controls were from Jackson Laboratories (Bar Harbour, Me., USA). C3H10T $\frac{1}{2}$ mesenchymal stem cells were obtained from American Type Culture Collection (Rockville, Md., USA). Pathogen-free eggs were purchased from Spafas Farms (Storrs, Conn., USA). All tissue culture supplies were from either Sigma or Gibco BRL Life Technologies (Grand Island, N.Y., USA). Transwell Culture Devices were from Millipore Corp (Medford, Mass., USA).

Production of Simple Transverse Fractures and Marrow Ablation

Eight- to ten-week-old male knock-out mice and matched control mice were used for this study. Closed, transverse, mid-diaphyseal fractures of the tibiae were generated by controlled blunt trauma using a modification of the technique developed for rats [Bonnarens and Einhorn, 1984; Hiltunen et al., 1993]. The tibia was chosen for use in the mouse versus the femur in the rat, since for this species it provides better anatomic features than the femur for the performance of the fracture procedure [Hiltunen et al., 1993]. Fracture stabilization by intramedullary fixation was carried out using the trocar of a 25-G and 23-G spinal needle.

Tibia marrow ablation was performed as described by Suva et al. [1993]. Briefly, mice were anesthetized by inhalation with isoflurane. A small incision along the medial aspect of the proximal muscle overlying the tibia was made and the muscle retracted to expose the bone. A small 1-mm hole was drilled through the cortex into the marrow space. A 23-G needle was then inserted the length of the marrow cavity and gently rotated to disrupt the marrow cavity. The marrow contents were removed by vacuum suction and the space flushed with sterile saline. Following ablation the site was closed with suture. The animals were permitted full weight-bearing and unrestricted activity after awakening from anesthesia for both models of bone repair. Mice were euthanized by cervical dislocation on various days over a 28-day period as reported in the results.

Histology

At the time of euthanasia, the fracture sites or marrow ablated bones were recovered and fixed for 3 days in 4% para-formaldehyde in phosphate-buffered saline at 4°C. Specimens were completely decalcified in Immunocal (Decal Chemical Corporation, Congers, N.Y., USA) and embedded in paraffin. The tissue was appropriately positioned in paraffin to make sagittal sections and cut at 6 μ m thickness. Sections were stained with hematoxylin and eosin.

Preparation of Avian Skeletal Cell Populations

Chondrocyte populations enriched in hypertrophic and non-hypertrophic cells were prepared from sterna of 17-day-old chick embryos and cultures were grown as previously described [Gerstenfeld et al., 1989]. Co-cultures were initiated with second passage chick embryo cephalic sterna chondrocytes.

Co-Culture with C3H10T $\frac{1}{2}$

C3H10T $\frac{1}{2}$ cells were plated at 2.5×10^4 cells per 33-mm diameter culture wells and maintained in DMEM 10% FBS for 24 h. At the end of this second 24-hour period, the medium was removed and the cultures were replenished with fresh medium. Co-culture was carried out using Millipore trans-well devices with 45 μ m size exclusion. Co-cultures were initiated 48 h after plating by placing the trans wells, pre-seeded with 2.5×10^5 avian effector cells, into the wells of the 33-mm dishes overlying the responder C3H10T $\frac{1}{2}$ cells. All measurements were made after 21 days of growth under co-culture conditions. All cell cultures were maintained in DMEM, 10% FBS unless otherwise noted.

RNA Analysis

RNA was isolated from cells or from knock-out and control animals. Animal tissues were collected in duplicate sets of pooled samples [n = 10 (days 1 and 3) and n = 5 (days 7, 14, 21 and 28)]. Bones were retrieved on the indicated days after fracture or marrow ablation and powdered under liquid nitrogen with a mortar and pestle.

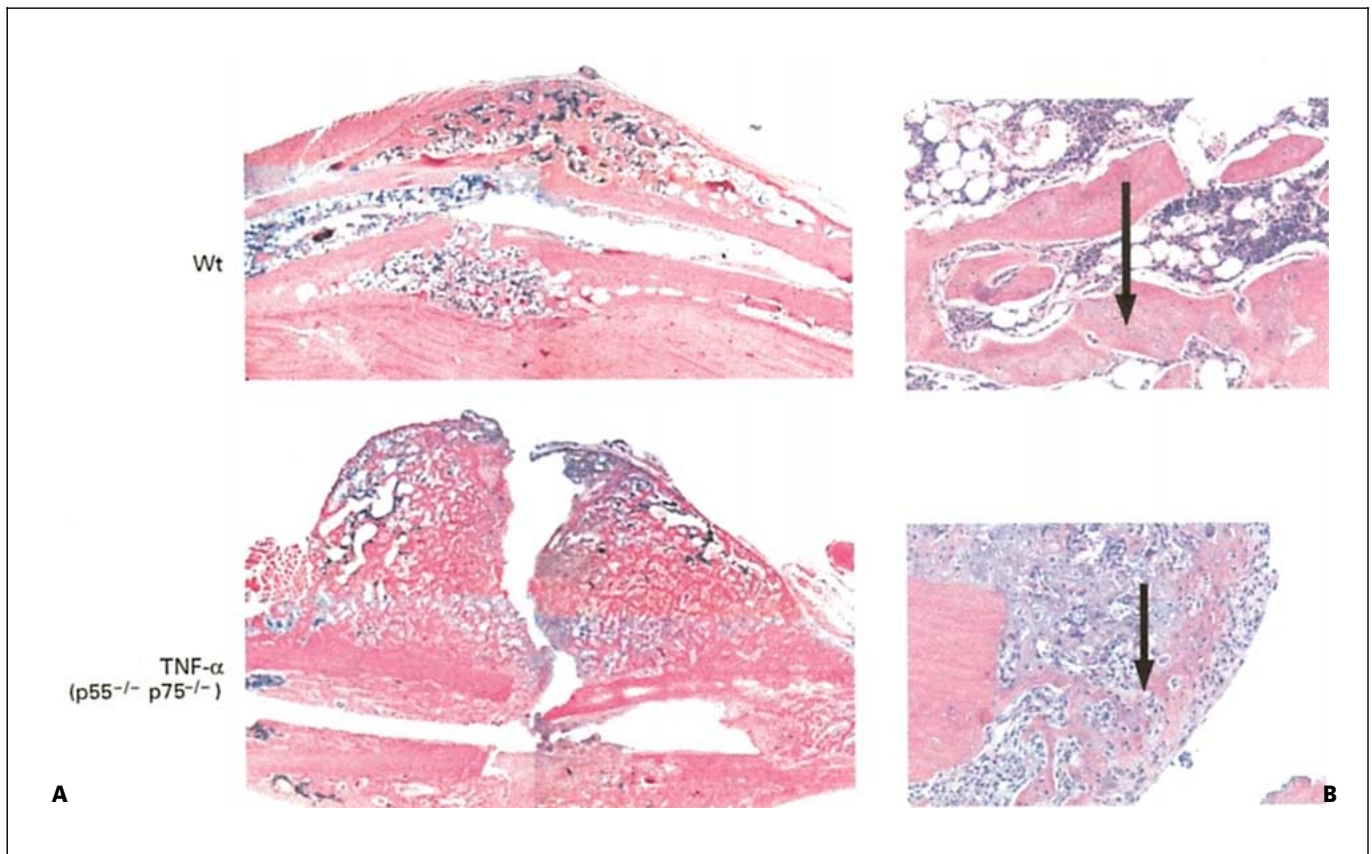


Fig. 1. Selected histological analysis of fracture repair of wild-type and TNF- α receptor TNFR p55^{-/-}/p75^{-/-} deficient mice. Days after fracture are indicated in the figure. **A** Low 50 \times magnification of the panoramic view of the fracture repair at day 21 after fracture. Note the dramatic differences in size of the calluses at 21 days. **B** Higher 200 \times magnification of 21 days at the fracture site highlighting the failure to induce periosteal induced membranous bone repair. The site of fracture is denoted with an arrow.

The total RNA was extracted from the powdered tissues or cells with Tri-ReagentTM (Molecular Research Center Inc., Cincinnati, Ohio, USA) as previously described [Toma et al., 1997]. RNA quantities were determined by OD_{260 nm} and sample integrity was monitored by visualization of ribosomal RNAs with ethidium bromide after denaturing RNA gel electrophoresis [Thomas, 1980].

RNase Protection Analysis

Messenger RNA expression during fracture healing was quantitatively assessed by ribonuclease protection analysis (RPA). Linearized plasmids for mouse collagen types I, II and X, osteocalcin, L32 and GAPDH were purchased from PharMingen Corp. (San Diego, Calif., USA). Single stranded ³²P-labeled cRNA probes were generated using RNA transcription kits purchased from PharMingen Corp. as per the manufacturer's instructions. RNase protection products were fractionated on a denaturing 6% acrylamide gel [Gerstenfeld et al., 1989]. The autoradiographic bands of the RNase protection products were quantified using an Alpha Innotech Image Analysis System, Alpha Innotech, Inc. Underexposed autoradiographic images were used for all analyses and the autoradiographic densities of the protected bands from each probe were measured. The quantities of indi-

vidual mRNAs for specific cytokines and extracellular matrix proteins were determined in the following manner. It was assumed that each probe had the same specific activity (i.e., the same ratio of radiolabeled nucleotides are incorporated for each run-off product) and that the annealing efficiencies between all the probes and their target mRNA sequences were the same under the conditions used in these studies. All data were normalized to either a so-called house-keeping gene such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or L32 mRNA.

Results

The role of TNF- α in endochondral bone formation was first assessed by comparing fracture repair in normal and knock-out mice deficient in both p55/p75 TNF- α receptors. Dramatically altered fracture healing was observed in the TNF p55^{-/-}/p75^{-/-} receptor deficient mice. Enlarged calluses were observed at 14, 21 and 28 days,

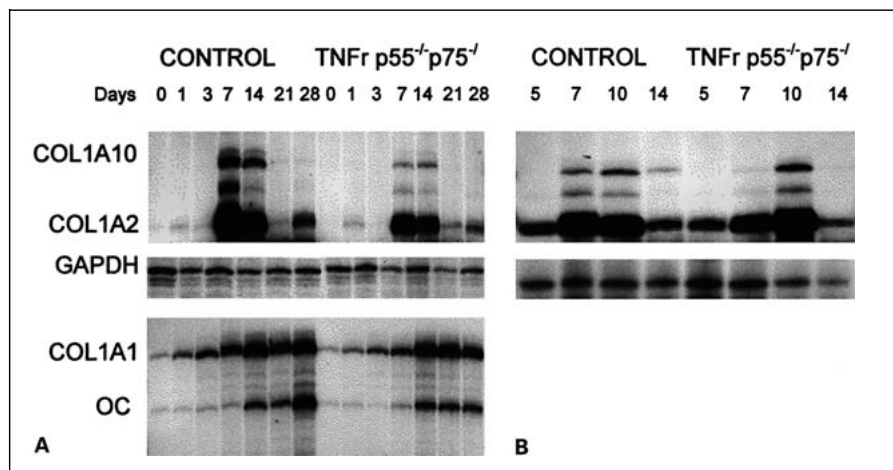
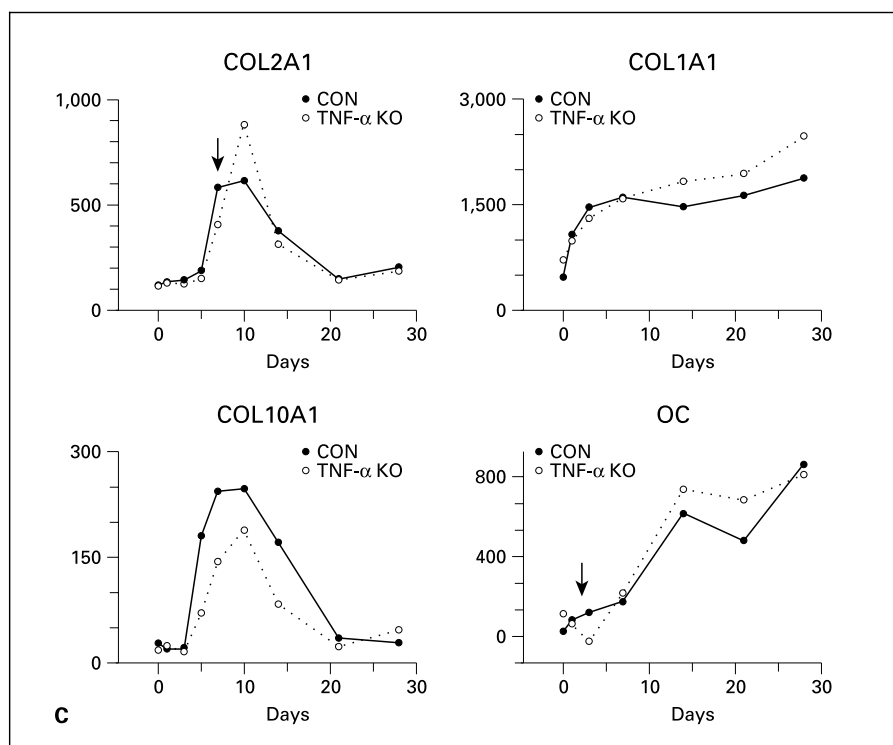


Fig. 2. Bone and cartilage gene expression during fracture repair in control and TNF- α receptor minus mice. **A, B** Representative RPA analysis of collagen types I, II, X, osteocalcin and GAPDH. Protections were carried out with 0.25 μ g of total RNA and the protected mRNA templates were resolved on an 8% denaturing sequence gel. The times after fracture and the nature of the mice from which the mRNAs were obtained are denoted in the figure. **C** Graphic analysis of the relative mRNA levels. Band densities were determined from several different autoradiographic exposures. Band densities were normalized to the ratio of the internal standards, GAPDH and L32. Values represent the absolute quantities of each of the ECM genes. Arrows in COL2A1 and OC panels note the delays in and bone and cartilage induction.



and the bones were more fragile to being re-broke during dissection in the TNFR p55^{-/-}/p75^{-/-} mice in comparison to the control animals. Histological analysis of the fracture sites confirmed the gross examination (fig. 1). The low magnification of the control and mutant animals at 21 days showed the enlarged nature of the calluses and a delay in removal of the calcified cartilage within the TNF- α receptor ablated mice. Higher magnification of the callus tissues at 200 \times showed in detail the cellular alterations in the repair tissues at day 21. One of the most striking features of the histological examination was the delay

in mesenchymal cell differentiation and the complete absence of the induction of the intramembranous bone formation on the periosteal surface. The failure to obtain intramembranous bone formation is better appreciated by comparison of the formation of an intramembranous bone bridge across the fracture gap seen in normal animals at 21 days compared to the lack of trabecular bridging seen at comparable post fracture times in the mutant animals (fig. 1, arrows). The delay in endochondral differentiation of the cartilage at later times was exemplified by the greater amounts of cartilage formed that were seen in

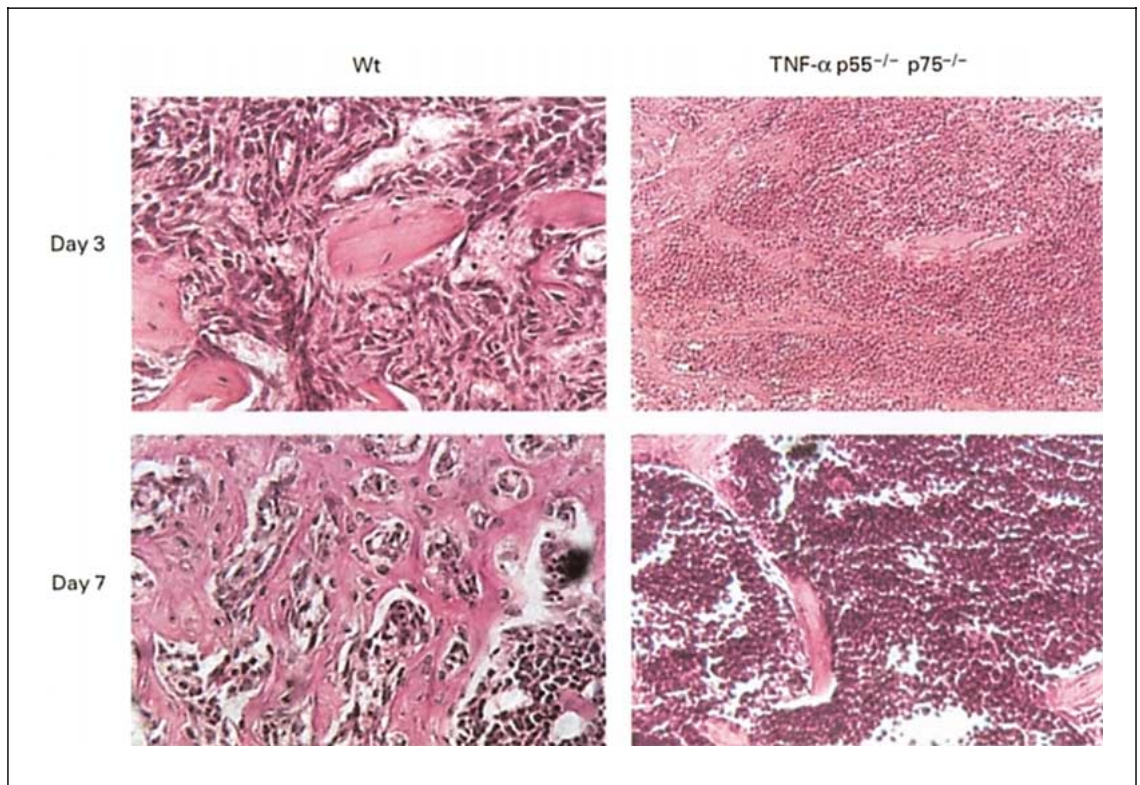


Fig. 3. Selected histological analysis of bone repair of wild-type (control) mice after marrow ablation. Days after marrow ablation are indicated in the figure. Low 40 \times magnification panoramic view of the bone repair at days 1, 3, 7, 21 and 28. Note the high degree of cellularity in the control tibia marrow space and lack of trabecular bone. At 1 day, newly formed bone is readily apparent after marrow ablation, while by day 7, the marrow space is completely filled with newly formed trabecular bone. At days 21 and 28, the newly made bone is remodeling and the marrow space is being restored.

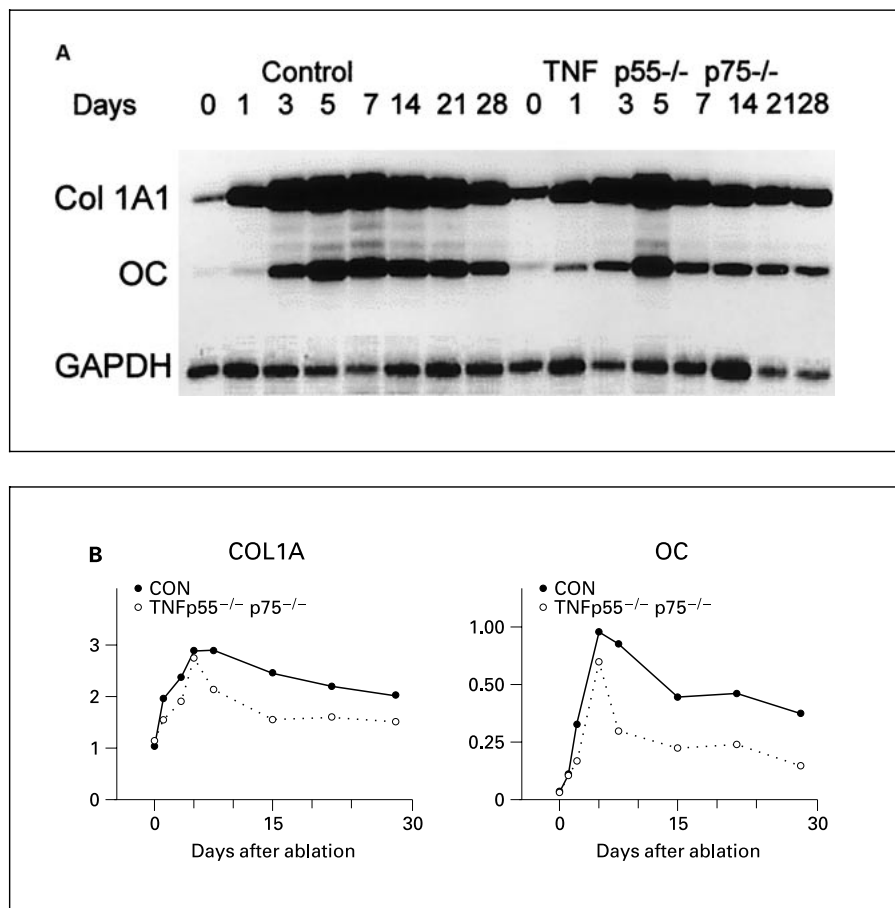
the callus and a delay in its hypertrophic maturation and removal in the mutant animals. Interestingly, at later times, even though intramembranous bone formation was absent in the mutant animals, there were a considerable number of osteogenic cells lining the cartilage tissue surfaces within the callus tissue.

The molecular processes of chondrocyte and osteoblast differentiation were assessed by analyzing the mRNA expression of various extracellular matrix gene products that are associated with the development of these tissues (fig. 2). These results demonstrated that the initial induction of bone formation, as exemplified by the expression of type I collagen and osteocalcin mRNA expression, was delayed but at later times the overall levels of these mRNAs were slightly higher ($\sim 20\%$) in the callus tissues from the mutant animals. The expression of collagen type II and X genes that are associated with growth and hypertrophic chondrocyte development, respectively, showed

overall slightly lower levels of expression at the two time points that were initially examined (fig. 2, left panel). While this initial examination appeared to be inconsistent with the histological findings in figure 1, examination of a much narrower temporal window between days 5 and 14 (fig. 2, right panel) when the endochondral process is maximally occurring better showed the delay and the elevated levels of type II collagen expression at day 10. The retarded progression of hypertrophic development is easily seen in the shift in peak mRNA expression seen for type II collagen and the altered ratios of collagen type II to type X mRNA expression (fig. 2B). These results overall then confirmed at a molecular level the delay in the endochondral progression during fracture healing in the absence of TNF- α signaling.

In order to determine if the absence of TNF- α signaling has a direct effect on osteogenesis, bone repair was next examined in the marrow ablation model. In this model,

Fig. 4. Bone gene expression during bone repair in control and TNF- α receptor minus mice after marrow ablation. **A** RPA analysis of collagen types I, II, X, osteocalcin and GAPDH. Protections were carried out with 0.5 μ g of total RNA and the protected mRNA templates were resolved on a 6% denaturing sequence gel. The times after fracture and the nature of the mice from which the mRNA were obtained are denoted in the figure. **B** Graphic analysis of bone and cartilage mRNA expression in control and TNF receptor deleted fracture repair tissues. The values were determined by digital image analysis of the scanned autoradiographs and correction of each mRNA value to GAPDH. The values for the individual genes are denoted in the figures. Graphic analysis of the ratio of osteocalcin to type II and collagen type II to type X mRNA expression are depicted in the last two panels. Legends denote the nature of the comparisons that are made. Solid line with squares = TNFp55^{-/-}/p75^{-/-}; triangles with dotted line = controls.



direct endosteal intramembranous bone formation occurs after the marrow has been cannulated. The role of TNF- α on bone formation was assessed histologically in normal and knock-out mice deficient in both p55/p75 TNF- α receptors (fig. 3). In control animals, bone formation is rapidly induced within the first 3 days and by 7 days extensive areas of new trabecular bone have been formed. In contrast, the hematoma has not been resolved and extensive amounts of necrotic tissue and cells were present at 3 days within the TNF- α receptor ablated mice. The mutant animals continued to show a delay in the induction of new bone formation and had a much smaller amount of newly formed trabecular bone still at 7 days. These results then showed that there was indeed a delay and diminished formation of new bone in the mutant animal.

Osteogenic differentiation was examined at a molecular level using the same RPA template set as was used in the analysis of fracture repair (fig. 4). As expected, no expression of the cartilage mRNAs was seen in this model. These results demonstrated that while the initial induc-

tion of osteogenic gene expression over the first 3 days was comparable in both the TNF- α receptor ablated mice and the control animals, at later time points there was a marked reduction in the expression of both osteocalcin and collagen type I mRNA expression. The reduced levels of expression were \sim 30% and \sim 50% for the osteocalcin and type I collagen mRNA expression, respectively, and the reduced levels of expression continued to persist throughout the remainder of the time period in which the repair process was monitored. These results further validated the observations of the reduced levels of cortical intramembranous bone formation that were primarily observed at the histological level in the fracture repair model at the early time points during fracture repair. A comparison of the results of the two repair models suggests that two separate processes mediate the induction of bone formation; one set of processes associated with the intramembranous induction on the cortical and endosteal surfaces, and a second set of processes linked to and possibly induced by signals from the endochondral cartilage.

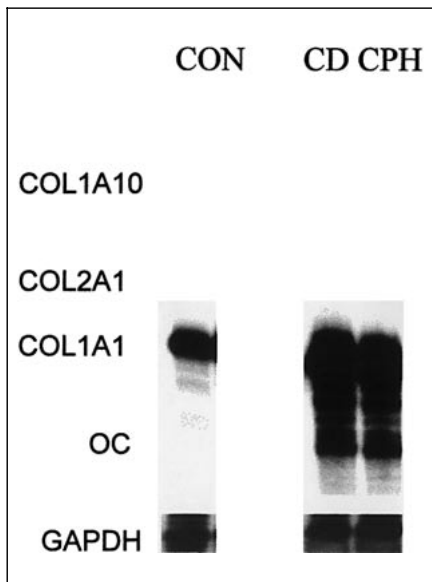


Fig. 5. Analysis of chondrogenic and osteogenic differentiation in control C3H10T $\frac{1}{2}$ cells or C3H10T $\frac{1}{2}$ co-cultured with avian embryo caudal (CD) and cephalic (CPH) sterna chondrocytes in DMEM, 10% FBS. Expression of collagen types I and II, and osteocalcin mRNAs were determined by quantitative RPA analysis. Representative autoradiographic images of the RPA products as resolved are shown. The mRNA species that each RPA product represents is denoted in the figure. Uniformity of gel loading is demonstrated by comparison of band to GAPDH.

One possible explanation for the variation in the apparent induction of osteogenesis in the two models of bone repair might be due to the expression of bone morphogenic signals by chondrocytes. Since in the absence of TNF- α signaling the apoptosis of endochondral chondrocytes was delayed, such a delay would lead to a persistence of any paracrine signals that are produced by these chondrocytes. In order to test the possibility that chondrocytes synthesized bone morphogenic signals, growth chondrocytes prepared from embryonic chick sterna were co-cultured with C3H10T $\frac{1}{2}$ mesenchymal stem cells. In previous studies, these cells had been shown to undergo differentiation into the major mesenchymal lineages including osteoblasts, chondrocytes, adipocytes and myoblasts [Constantinides et al., 1978; Ducky et al., 1997; Denker et al., 1999]. The results of this study are seen in figure 5. Upon co-culture with either of the chondrocyte cell populations, the cultures became multilayered and dense nodular areas were observed with morphology very similar to the morphology seen in primary osteoblast cultures [Gerstenfeld et al., unpubl. data]. The induction of both chon-

drogenic and osteogenic differentiation in individual cultures was examined using RNA protection analysis with the same template set containing probes that detected mRNAs expressed by either osteoblasts (collagen type I and osteocalcin) or chondrocytes (collagen type II, collagen type X). Interestingly, chondrogenic differentiation, as assessed by collagen type II and type X expression, was not observed under any of the culture conditions tested in these studies. In other studies by other groups using BMP 2 [Ducky et al., 1997; Denker et al., 1999] and our own studies in which BMP 7 was used, both chondrogenic (data not shown) and osteogenic differentiation was observed indicating that the morphogens produced by the chondrocytes selectively promoted osteogenic differentiation of these cells.

Discussion

The role of TNF- α in skeletal tissue repair was assessed in two separate experimental models: marrow ablation and fracture. Both models showed delayed healing in the TNF- α receptor deficient knock-out mice. These *in vivo* findings clearly demonstrate that TNF- α plays a primary role in initiating or maintaining the processes of bone repair. While intramembranous repair was impaired in both models, the primary defect in the fracture model was seen in the failure to resorb the endochondral cartilage tissue. The slower turnover of the cartilage tissue is most likely due to the primary role that TNF- α plays in stimulating chondrocyte apoptosis [Aizawa et al., 2000]. This conclusion leads to consideration of a number of potential mechanisms by which TNF- α controls the removal of the calcified cartilage. These considerations include the functional role that TNF- α plays in regulating chondrocyte hypertrophic maturation, expression of pro-resorptive signals, effects of chondrocyte apoptosis, and regulation of osteogenic differentiation.

Previously, it had been suggested that that mesenchymal stem cell recruitment into the osteogenic lineage during endochondral bone formation is dependent upon prior chondrogenic differentiation and locally produced soluble signals elaborated by chondrocytes [Gerstenfeld and Shapiro, 1996]. In support of this hypothesis, extensive new data have emerged with the use of genetically engineered mice that have further elucidated the relationships between cartilage and bone development during embryogenesis. Ablation of the PTHrP gene causes an osteochondrodysplasia, primarily manifested by acceleration in chondrocyte hypertrophy [Karaplis et al., 1993]

and leads to precocious osteogenesis. In contrast, overexpression of PTHrP causes delayed chondrocyte differentiation with greatly diminished osteogenesis. Other studies have shown that Indian hedgehog protein is part of a negative feedback loop in which Ihh production by prehypertrophic chondrocytes induces PTHrP expression, thereby inhibiting the progression of chondrocyte differentiation [Karp et al., 2000]. Interestingly, ablation of the Ihh gene alone, beyond leading to changes in growth and chondrocyte differentiation, also leads to a failure of osteoblast development [St-Jacques et al., 1999; Bucay et al., 1998]. Both of the current in vivo studies and the supporting in vitro studies provide further data that draws a linkage between chondrogenic progression in endochondral bone formation and subsequent osteogenesis. The studies presented here provide additional data demonstrating how a deficiency in chondrogenic differentiation leads to subsequent downstream alterations in osteogenesis. In addition, these studies suggest a developmental mechanism by which mesenchymal lineage recruitment into a variety of differentiation pathways is constantly modified according to the local cellular composition. Thus, the effects that TNF- α has in an endochondral environment produce

very different outcomes on osteogenic differentiation than in an endosteal environment where only intramembranous bone formation occurs.

The effects of the absence of TNF- α signaling on bone repair in response to marrow ablation suggest that it also has a direct role in vivo on osteogenic differentiation during intramembranous bone repair. It is unclear at present, though, by what molecular mechanisms TNF- α signaling is effecting osteogenesis in the marrow ablation model, since a number of studies suggests that TNF- α inhibits osteoblast differentiation when it is administered to osteoblast-like cells in vitro [Gilbert et al., 2000; Horiguchi et al., 2000]. One possible mechanism may be that TNF- α attenuates the inflammatory processes after injury. In the absence of such signals in the ablated animals, other alterations in the network of immune cytokines then inhibit osteogenesis. Irrespective of the mechanisms by which TNF- α signaling mediates its effects of osteogenesis during bone repair, the current studies provide further evidence of the central role that TNF- α signaling plays in regulating several different aspects of both intramembranous and endochondral bone repair.

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