

Exogenous transforming growth factor beta 1 alone does not improve early healing of medial collateral ligament in rabbits

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Objective: To determine whether transforming growth factor beta 1 (TGF- β 1) improves early ligament healing. **Design:** Experimental, controlled study of medial collateral ligaments (MCLs) in rabbits' knees. **Setting:** Research laboratory. **Subjects:** Sixteen skeletally mature, New Zealand White female rabbits. **Interventions:** Ten rabbits had a standardized gap injury made in the MCL of both knees. Three weeks later, a second operation was performed to inject 7 μ g of TGF- β 1 in a carrier solution into the right knee MCL, while the left knee MCL was injected with carrier alone. The rabbits were killed 3 weeks after the injection of TGF- β 1 (6 weeks after the original injury). Six of the rabbits (12 knees) had no operation on the MCL and served as external normal controls. **Outcome measures:** Biomechanical measures of the femur-MCL-tibia complex. Histologic evaluation of MCL cell and matrix organization. Transmission electron microscopy measures of MCL fibril diameters. **Results:** There were no statistically significant differences in the biomechanical measures, fibril diameter distributions and histologic evaluation of the injured MCLs treated with TGF- β 1 or carrier alone. Both groups of injured MCLs were significantly different from normal MCLs. **Conclusions:** The results indicate that the dosage and route of delivery of TGF- β 1 did not lead to overt improvement in the healing of the injured MCL. Whether different doses or delivery methods, alone or in combination with TGF- β 1, or other growth factors would lead to improvement remains to be determined.

Objectif : Déterminer si le facteur de croissance transformant bêta 1 (TGF- β 1) améliore la guérison précoce des ligaments. **Conception :** Étude contrôlée expérimentale portant sur les ligaments latéraux internes (LLI) de genoux de lapins. **Contexte :** Laboratoire de recherche. **Sujets :** Seize lapines New Zealand White dont le squelette était à maturité. **Intervention :** On a pratiqué une brèche normalisée dans le LLI des deux genoux chez 10 sujets. Trois semaines plus tard, on a pratiqué une deuxième intervention pour injecter dans le LLI du genou droit 7 μ g de TGF- β 1 dans une solution de support tandis que l'on a injecté dans le LLI du genou gauche la solution de support seulement. Les lapines ont été mises à mort trois semaines après l'injection de TGF- β 1 (six semaines après le traumatisme initial). Six des sujets (12 genoux) n'ont pas subi d'intervention au LLI et ont servi de témoins normaux externes. **Mesures de résultats :** Mesures biomécaniques du complexe fémur-LLI-tibia. Évaluation histologique des cellules du LLI et structure matricielle. Mesures du diamètre des fibrilles du LLI effectuées par microscopie électronique à transmission. **Résultats :** Il n'y avait pas de différences significatives sur le plan statistique entre les mesures biomécaniques, les distributions du diamètre des fibrilles et l'évaluation histologique des LLI blessés et traités au moyen du TGF- β 1 ou de la solution de support seulement. Les deux groupes de LLI blessés présentaient des différences significatives par rapport aux LLI normaux. **Conclusions :** Les résultats indiquent que la dose et la voie de distribution du TGF- β 1 n'ont pas entraîné d'amélioration évidente de la guérison du LLI blessé. Il reste à déterminer si des doses différentes ou des méthodes de distribution différentes, seules ou combinées au TGF- β 1, ou à d'autres facteurs de croissance, entraîneraient une amélioration.

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It has been suggested that the medial collateral ligament (MCL) heals by "scar" formation rather than by true ligament regeneration.¹ Untreated ligament scar has been shown to be biochemically different from normal ligament tissue, even at long-term follow-up, with particular changes in both quantity and quality of collagen.¹ The diameters of collagen fibrils in ligament scars also remain smaller than those of normal ligaments even after 2 years of healing.² Ligament scars have mechanical properties that are inferior to normal ligament substance.¹

In recent years, several investigators have begun to explore potential means of manipulating scar quantity and quality with peptide growth factors.³⁻¹¹ More specifically, transforming growth factor beta 1 (TGF- β 1), a 25 kD homodimeric protein,^{2,12,13} has been shown to stimulate the proliferation of fibroblasts¹⁴⁻¹⁸ and synthesis of extracellular matrix proteins *in vitro*.^{14,15,19,20} *In vivo*, in an incisional skin wound model in rats, 2.0 μ g of TGF- β 1 was shown to markedly increase early wound strength, presumably because of marked increases in collagen deposition at that site.²¹ Conti and Dahners²² compared the effects of exogenous TGF- β 1 on MCL healing in a rat model and suggested that at 12 days only TGF- β 1 (1 μ g) improved the early mechanical properties of these healing ligaments. In our laboratory, Murphy and colleagues²³ showed that 2.5 ng/mL of TGF- β 1 added to 3-week-old ligament scar explants increased collagen secretion and modulated the ratio of type I and type III collagen secretion to be closer to that found in normal ligament. On the basis of these studies, we speculated that TGF- β 1 alone may be a good candidate for stimulating ligament healing *in vivo*. Previous work in our laboratory using lower doses of TGF- β 1 (50 ng and 200 ng) to augment MCL scar quality showed no effect on measured mechanical and structural properties of the bone-MCL-bone complex.^{24,25}

In the present study, based on the range of apparently successful doses used by others,^{21,22,26} a higher dose (7 μ g) of TGF- β 1 was applied to a well-characterized model of MCL gap scars *in vivo*. Our hypothesis was that the higher dose of TGF- β 1 would stimulate scar remodelling and produce improved scar quality.

Materials and methods

In 16 skeletally mature New Zealand White rabbits (Riemens Fur Ranch, St. Agatha, Ont.), 12 months old and weighing 5.1 (0.5) kg (mean [and standard deviation]), both hind limbs were shaved and prepared with iodine. Under general anesthesia, MCL gaps were made in both legs of 10 rabbits in the same manner.²⁷ The MCLs were exposed by a longitudinal incision through overlying fascia. A 2-mm segment of the MCL mid-substance, centred just distal to the joint line, was removed. A small gap thus formed between opposing ligament ends. The 4 corners of these cut ends were then marked with 6-0 nylon sutures to assist in identifying the area of the healing MCL. These animals were allowed to move around unrestricted in cages measuring 65 \times 45 \times 30 cm. Three weeks later, again under general anesthesia, skin over both knees was reopened

with the same techniques as the first operation; however, fascia covering each healing MCL was left intact. Under direction vision, a single injection of 7 μ g of "recombinant human" TGF- β 1 diluted in 1% rabbit serum albumin (RSA) in 100 μ L of phosphate-buffered saline was instilled directly into the right MCL scar tissue through the overlying fascia using a 29-gauge needle. In the left leg, which was exposed similarly, 1% RSA in 100 μ L of phosphate-buffered saline was injected into the MCL through the overlying fascia as a control. Skin incisions were resutured and the animals allowed to return again to full cage activity. It should be noted that before this experimental series, a pilot study had been carried out in which 100 μ L of methylene blue dye with 1% RSA had been injected in the same manner. It was confirmed that injected solution had stayed in and around the gap scar tissue (Fig. 1). Three weeks after the injections (6 weeks after the original gap injury), all experimental animals were killed. The MCLs from both legs were allocated to either biomechanical testing (8 rabbits) or histologic and electron microscopic evaluation (2 rabbits). Twelve normal MCLs of 6 rabbits were used as an external normal control group and were studied in parallel.

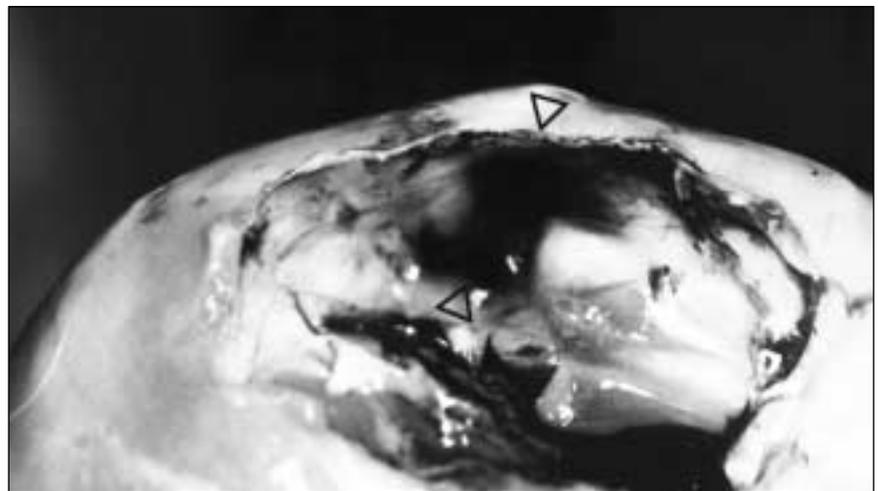


FIG. 1. Methylene blue dye with 1% rabbit serum albumin (100 μ L) injected directly into the medial collateral ligament scar through the fascia remained in the gap scar tissue with minimum leakage (arrowheads).

Biomechanical testing

Biomechanical studies followed previously published protocols from our laboratory.^{28,29} All experimental and control MCL specimens were tested fresh. On the day of testing, the animals were killed and their hind limbs disarticulated. All soft tissue, including muscles and fascia, was removed from the femur and tibia, excluding the collateral ligaments, the cruciate ligaments and the menisci. Bones were transected 4 to 5 cm from the joint line. The tibia was then "potted" in a specially designed clamp, using polymethylmethacrylate (PMMA), and this clamp was attached to the actuator of the MTS Model 490 TestStar II materials testing system (MTS Systems; Eden Prairie, Minn.). The knee flexion angle was adjusted to 70° and the cross-head lowered to place the femoral end of the specimen into a second clamp, where it was fixed with PMMA. The longitudinal axis of the MCL was aligned with the load axis of the testing system.

The knee joint was taken through 2 cycles of loading and unloading at an extension rate of 1 mm/min, first compressing the joint by 5 N and then reversing to a tensile force of 2 N. Joint laxity was determined by measuring actuator displacement between these loads. With the knee joint at deformation 0 (neutral position), the remaining soft tissue was removed, isolating the MCL. The knee joint was again taken through 2 cycles of compression (5 N) and tension (2 N). MCL laxity was determined by the amount of displacement from the point of compression to the point at which the MCL takes up any detectable load. The actuator position where the isolated MCL first registered a tensile load (0.1 N) was then defined as "ligament zero" and was used as the starting position for all subsequent mechanical tests on that ligament. A small portion of each medial femoral condyle was then removed to allow in situ mea-

surement of midsubstance MCL cross-sectional area with a previously described measuring device.³⁰ A specially designed humidity chamber was quickly closed around the clamping system and warmed to 36°C.

Each femur-MCL-tibia complex was cycled 33 times between "ligament zero" and a fixed deformation of 0.7 mm (approximately 3% average strain for the average mature rabbit ligament) at an extension rate of 10 mm/min. Cyclic load relaxation was measured as the difference between the peak loads of the first and tenth cycles divided by the peak load of the first cycle, expressed as a percentage. Immediately after this cyclic test, specimens were distracted at 10 mm/min to 0.7 mm from "ligament zero," where they were held and allowed to load-relax for 1200 seconds. Static load relaxation was defined as the difference between the initial peak load and the load measured after 1200 seconds divided by the initial load, also expressed as a percentage. Ligaments were then returned to ligament deformation of 0 mm and loaded to failure at an extension rate of 2 mm/min. The modes of ligament failure were observed visually and recorded.

MCLs were exposed immediately after sacrifice of the animal. Areas of scar tissue between marker sutures were removed and cut longitudinally in the frontal plane into 3 parts. The middle piece was used for transmission electron microscopy and the other pieces were used for light microscopy.

Histologic evaluation

Specimens were fixed overnight in 10% neutral buffered formalin and then processed for embedding in JB4 plastic (Polysciences, Warrington, Penn.). A microtome (Leica Super-cut model 2065) was used to cut 3.0-mm sections. All sections were stained with hematoxylin and eosin. Qualitative examination under light microscopy was performed to deter-

mine cellularity, vascularity, fibre arrangement and relative amounts of matrix present.

Measurement of collagen fibril diameters

For measurement of collagen fibril diameters by transmission electron microscopy, the method of Frank and associates was used.^{31,32} The central strip of tissue was sliced into small longitudinal slivers (approximately 0.2 mm wide) with a razor blade and fixed for a minimum of 2 hours in modified Karnovsky's fixative.³³ Samples were then rinsed with 0.1 M cacodylate buffer containing 5 mM calcium at pH 7.4 and post-fixed for 1 hour in 1% osmium tetroxide in the same solution. They were then dehydrated in an ascending ethanol series and embedded in low viscosity Spurr's resin, with careful attention to orientation. Silver-gold sections approximately 100 nm thick were then cut with a diamond knife on a Reichert OM-U3 ultramicrotome (Cambridge Instruments, Toronto), mounted on 300-mesh uncoated copper grids and stained with aqueous uranyl acetate and lead citrate.³⁴ Collagen fibril diameter was measured after temperature stabilization on a Hitachi H-7000 electron microscope operating at 75 kV. Micrographs of fibril cross-sections were taken at $\times 30\,000$ magnification in representative areas of the tissue. Regions containing cell processes, resin fracture or defined cutting artifacts were avoided. Negatives (8.9×10.2 cm) were analysed individually on an image processing system (IBAS, Kontron Electronics, Echting/München, Germany) with a resolution of 512 pixels. A minimum of 1000 transversely cut fibrils was measured for each ligament.

Statistical analysis

Differences in the biomechanical and fibril diameter measured for the TGF- $\beta 1$ applied group, the control

group and the external normal controls were analysed by one-way analysis of variance, followed with Fisher's PLSD post-hoc test. Statistical significance was set at *p* less than 0.05 for all comparisons.

Results

Biomechanical testing

One rabbit scheduled for biomechanical testing died before evaluation. The remaining 7 rabbits were tested. The results of biomechanical testing for the TGF-β1-injected MCLs and the contralateral control MCLs are summarized in Table 1. There were no statistical differences between these 2 groups for any parameter. Compared with external nor-

mal control MCLs, both groups had significantly larger MCL scar cross-sectional areas, lower failure loads and failure stresses along with altered viscoelastic properties (cyclic and static load relaxation) than the normal MCLs (*p* < 0.05).

Light microscopic evaluation

Typical ligament photomicrographs are shown in Fig. 2. No differences were detected between the TGF-β1-injected scars and the contralateral control scars in regard to cellularity and vascularity. MCL scars of both groups were hypercellular, with increased vascularity. Some qualitative differences in matrix were seen, with collagen fibrils being slightly less dense and more disorga-

nized in the TGF-β1 scars than in the contralateral control scars.

Transmission electron microscopic evaluation

The distribution of collagen fibril diameters in healing ligaments are shown in Fig. 3. Small collagen fibrils 48 to 96 nm in diameter were predominant in both the TGF-β1-injected and control MCLs. The pattern of fibril diameter distributions was similar in these 2 groups and were clearly different from those of normal control MCLs.^{2,31}

Table 1

Biomechanical Properties of Femur-Medial Collateral Ligament (MCL) Scar-Tibia Complex for Rabbit MCLs Injected With Transforming Growth Factor Beta 1, Carrier Alone (Control) or Neither (External Normal)

Property	TGF-β1, 7 μg (n = 7)	Control (n = 7)	External normal (n = 12)
Cross-sectional area, mm	7.6 (4.0)	8.9 (1.8)	3.9 (0.2)
MCL laxity, mm	1.27 (0.42)	1.17 (0.51)	0.28 (0.19)
Cyclic load relaxation, %	46.4 (7.4)	47.3 (4.8)	20.3 (3.2)
Static load relaxation, %	69.3 (16.7)	66.6 (7.7)	36.9 (5.0)
Stiffness, N/mm	40.0 (19.0)	47.9 (15.7)	107.1 (9.6)
Failure load, N	77.3 (45.3)	105.9 (42.0)	368.6 (48.5)
Failure stress, MPa	10.5 (3.5)	12.1 (4.7)	95.2 (12.4)

Values are means (and standard deviations). Note: there are no significant differences between TGF-β1-treated and control groups for any parameters. All of the experimental groups are significantly different from external normal controls for all parameters (*p* < 0.05).

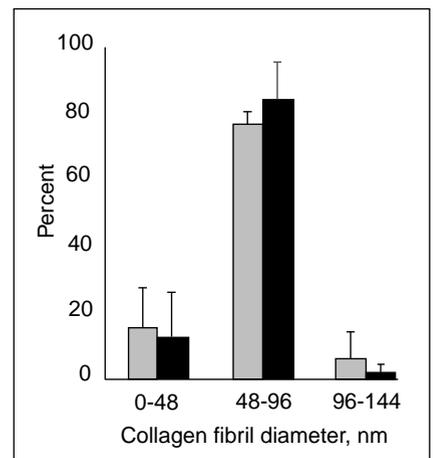


FIG. 3. Distribution of collagen fibril diameter for the medial collateral ligament scars injected with transforming growth factor beta 1 (7 μg, shaded bars) and contralateral control scars injected with only carrier (black bars).

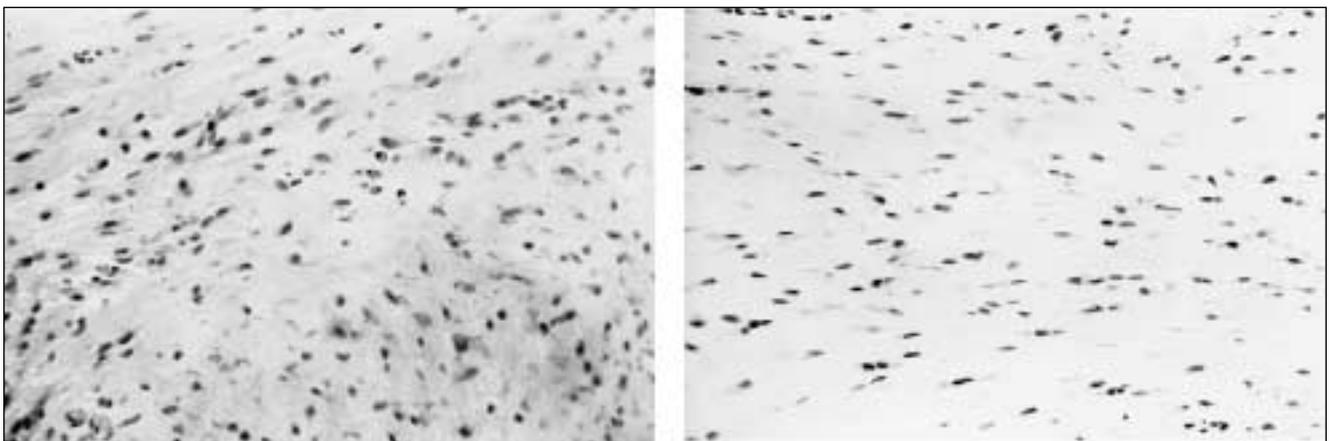


FIG. 2. Histologic appearance of representative sections of scar tissue after a single injection of 7 μg of transforming growth factor beta 1 (left) and the contralateral healing control (carrier injected) (right) at 6 weeks after injury (3 weeks after injection). Note the slight differences in the arrangement and relative amounts of collagen (hematoxylin-eosin, original magnification ×312).

Discussion

Despite the apparent stimulatory effects of TGF- β on wound healing²¹ and MCL strength in rats,²² results of the present study showed no beneficial effects on early scar quantity or quality of rabbit MCL *in vivo*. If anything, biomechanically, scars injected with a higher dose (7 μ g) of TGF- β 1 showed a trend to being weaker at the interval studied (Table 1). These findings mirror previous work done in our laboratory in which lower doses of TGF- β 1 (50–200 ng) did not improve early MCL scar healing biomechanically.^{24,25} Results of transmission electron microscopy evaluations similarly showed no effect of the TGF- β 1 in that the diameters of the collagen fibrils were comparable in both TGF- β 1-injected and contralateral control scars (Fig. 3). Histologic evaluations showed only a slight difference, with collagen fibrils being slightly less dense and more disorganized in the TGF- β 1 scars than in contralateral controls (Fig. 2). Loose, disorganized collagen can represent mechanical “flaws” in ligament scars and cause them to be weak.³⁵

Conflicting results regarding the effects of TGF- β on ligament healing have been reported. Positive effects have been reported on tissue explant culture DNA synthesis and collagen metabolism and on early rat MCL scar biomechanical properties.^{22,23,26} However, several authors have shown no effect or speculated about the detrimental effects of TGF- β on biomechanical healing properties of MCL scars or tissue culture when TGF- β was used alone or combined with other growth factors.^{4,15,36,37}

Exactly why the application of the recombinant TGF- β 1 failed to improve ligament scar formation and remodelling *in vivo*, despite its effectiveness on ligament scars *in vitro*,²² is debatable. The strongest possibility is that endogenous TGF- β 1 may already be sufficient to optimize scar

formation in this extra-articular environment. Lee and associates³⁸ have shown TGF- β 1 is expressed weakly at 1 and 3 days and strongly at 7 and 10 days in the pericellular region of rabbit MCL scars, but not thereafter. Natsu-ume and colleagues³⁹ showed that TGF- β 1 was intensely expressed in patellar tendon wounds for up to 28 days after injury. It has also been reported that expression of TGF- β 1 messenger RNA (mRNA) increased 9-fold within 24 hours after wounding and remained high for several days after injury in mouse skin wounds.⁴⁰ Recent work in our laboratory by Sciore and colleagues⁴¹ has shown high levels of mRNA for TGF- β 1 in 3-week scars. From these collective results, there is no doubt that TGF- β 1 likely plays an important role in the early healing responses of skin, tendons and ligaments. However, it may be that endogenous levels of TGF- β 1 already are sufficient for collateral ligament healing and additional exogenous TGF- β 1 does not increase this effect.

A second possibility to explain the inability of exogenous TGF- β 1 to improve MCL healing is that TGF- β 1 may have either become inactivated or become bound to scar matrix molecules, preventing any detectable activity in this healing model. It has been shown that the molecular form of [¹²⁵I]rhTGF- β 1 remains in wound sites for only a few days.²⁶ This short duration of potential effectiveness may be insufficient for ongoing modification of scar quality with a single injection. Alternatively, there may be more extensive mechanisms of inactivation and removal *in vivo*.

A third possibility is that the doses of TGF- β 1 chosen for injection may have been inappropriate. TGF- β 1 has been suggested to improve wound healing in a dose-dependent manner. In the model of glucocorticoid-induced wound-healing deficit, the breaking strength of TGF- β -treated wounds was strongest at doses of 1.0 μ g.¹⁰ In radiation-

impaired wound models, the breaking strength of wounds was strongest with 5 μ g of TGF- β .⁴² The optimal dose used to improve early MCL healing in rats was 1 μ g.²² On the other hand 20 μ g of TGF- β 1 has been shown to be inhibitory.⁴² Based on this spectrum, a dose of 7 μ g, which was more than 100 times greater than the lowest dose used in our previous studies, was used.^{24,25} Thus, the dose chosen for study, 7 μ g/animal, is lower than inhibitory concentrations but considerably higher than lower doses also reported to be ineffective in improving ligament scar quality.

A fourth possibility is that the timing of TGF- β 1 application may not have been optimal. In the low-dose study, TGF- β 1 was applied over 3 weeks from the time of injury to the time of sacrifice, showing no effect in biomechanical properties.^{24,25} In the present higher dose study, TGF- β 1 was applied in a single injection at a point of known peak cellularity of fibroblasts¹ in an attempt to achieve maximal scar stimulation, but this also showed no effects. From these results, it would appear that administration of TGF- β 1 alone within the first few weeks of collateral ligament healing in healthy adult animals would have no effect on scar quality.

A fifth possibility considers the method of growth-factor delivery. Our studies used rabbit serum albumin diluted in phosphate-buffered saline to deliver the growth factors with either a single injection or continuous administration through an osmotic pump. Another group that did not report positive effects on MCL healing with TGF- β used slow release pellets,⁵ whereas the group that reported positive effects of TGF- β on MCL healing used a rat tail collagen emulsion delivery vehicle.²² The elution rates of (active) TGF- β , and potential sources of other growth factors or stimulatory or inhibitory factors in the delivery vehicles, could all have an impact on the success or failure of a growth factor to alter MCL healing.

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SESAP Questions Questions SESAP

Category 11, Items 33 to 35

33. Reliable screening technique
34. Require(s) mediastinal abnormalities to detect significant injury
35. Equivocal findings mandate aortography

For each of the numbered items above, select the applicable lettered word or phrase below

- (A) Helical computed tomography for traumatic aortic disruption
 (B) Chest x-ray for traumatic aortic disruption
 (C) Both
 (D) Neither

For the answers and a critique of items 33 to 35 see page 362.

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