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ORIGINAL ARTICLE

Laser burn wound healing in naso-labial region of fetal and neonatal mice

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OBJECTIVE: To investigate the characteristics of wound healing in the mouse naso-labial region in both the fetal and neonatal stages, histological and immunohistochemical analyses were performed using a newly established laser burn wound healing system.
MATERIALS AND METHODS: Fetal mice at embryonic day 14 (E 14) were wounded as a model of fetal wound healing. To compare it, neonatal mice at day 5 after birth (d 5) were adopted as a model of neonatal wound healing. The healing process was examined by van Gieson staining and immunohistochemistry for fibronectin and tenascin.
RESULTS: Relatively large damage remained after wound healing even in fetal mice. In both types of wound healing, rapid regeneration of muscle tissues were observed. Fibronectin and tenascin immunostaining was detected not only in wound healing region, but also in the endomysium of regenerating muscle tissues. Especially, tenascin showed a restricted expression pattern.
CONCLUSIONS: Rapid regeneration of muscle tissues in the naso-labial region in both the fetal and neonatal mice seemed to leave relatively large damage even in the fetal wound healing. Contracted force exerted by muscle tissues may be a reason for this phenomenon. Fibronectin and tenascin were closely related to the wound healing process including muscle regeneration in this region.
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Keywords: wound healing, laser, muscle, fibronectin, tenascin, immunohistochemistry

Introduction

Scar formation after cheiloplasty or palatoplasty on a cleft lip and palate are known to cause various problems for the patients, such as mid-facial growth retardation, narrower upper dental arch, flat palate and lingual tipping of the maxillary anterior teeth (Huang *et al*, 2002; Honda *et al*, 2002; Berkowitz *et al*, 2004). On the other hand, no scar formation has been demonstrated in the wound healing of the fetal skin (McCallion and Ferguson, 1996). Namely, favorable results could be expected in terms of facial esthetics and maxillofacial growth by the application of fetal surgery.

Studies on the morphological and histological effects of fetal surgery in the naso-labial region have been done using large experimental animals such as rabbits (Longaker *et al*, 1990a; Dodson *et al*, 1991; Stern *et al*, 1992) and sheep (Stern *et al*, 1993; Stelnicki *et al*, 1999). According to the results of these studies, scar formation was not observed; however, at the same time, deformation (shortening) of the lip and some inhibition of mid-facial growth were also observed. These results seemed to be caused by a factor other than scar formation and may be location-specific characteristics of the naso-labial region, since other fetal wound healing studies dealing with dermal tissue of the back or limbs demonstrated neither scar formation nor deformation in the corresponding regions (Longaker *et al*, 1990b). Since the lips, in particular, are specially differentiated organs for functions such as mastication and speech, they have a specific tissue structure that differs from other ordinary skin, for example skin of back, as being surrounded by numerous muscle groups such as the orbicularis oris muscle and muscles of facial expression. We hypothesized that muscle regeneration of this region may have significant influences on the wound healing processes.

Suzuki *et al* (2004) established a fetal mouse wound healing model of the naso-labial region using argon laser. This laser wounding model is different from other wound healing models such as incisions in thermal injury induced and in the larger area of tissue necrosis, but this model can easily make fetal wound without disrupting amniotic sacs and dynamic histological changes can be observed in severe injury rather than

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simple incision. Therefore, we have accepted this experimental model to observe the characteristics of the wound healing process.

Meanwhile, the temporal and spatial expression of the extracellular matrix, especially, fibronectin (FN) and tenascin (TN) is important in many phases of wound healing (Whitby and Ferguson, 1991; Whitby *et al*, 1991; Kanno and Fukuda, 1994). No previous studies, however, described the relationship between FN and/or TN and muscle regeneration in wound healing process, although no TN expression is reported in normal endomysium, perimysium and epimysium (Järvinen *et al*, 2000).

To confirm the above hypothesis, we investigated the characteristics of the healing process of a laser burn wound in the naso-labial region with reference to FN and TN expression in both fetal and neonatal mice.

Materials and methods

All animals were maintained in the animal research center of the Tokyo Medical and Dental University and the procedures conformed to the guidelines determined by the University Animal Care Committee. Research protocols conformed to NIH guidelines as stated in the 'Principles of Laboratory Animal Care' (NIH publication no. 86–23, revised 1985).

Experimental procedure

The wounds for two groups of ICR mice were studied. Postnatal wounds were created on day 5 after birth (d 5) (21 samples) and fetal wounds were created on embryonic day 14 (E 14) (12 samples) according to the results from a previous study (Suzuki *et al*, 2004): note, as preliminary experiments, we obtained high mortality in less than E 14 postoperatively, and scar tissue remained in fetuses operated in later period of pregnancy as well as postnatal, in addition, since mice d 5 are relatively young and easy to handle. After the operation, the postnatal and pregnant mice were individually caged and postoperatively fed a standard rodent chow and water *ad libitum*.

In fetal wounded group, the maternal mice were administered ritodrinehydrochloride (Utemenal®, Sankyo, Tokyo, Japan) (0.003 mg g^{-1} body) as tocolytic and anesthetized with a sodium pentobarbital (Somunopentyl®, Schering Plough, Osaka, Japan) (0.03–0.05 mg g^{-1} body) by preoperative intrapenitoneal administration. The fetus along with the amniotic membrane was exposed. An argon laser (AC-2300; Nidek, Tokyo, Japan) then irradiated the left site of the naso-labial region five or six times through the amnion until the site became white. The pulse duration of 0.5 s, the wave length of 488/514 nm and the power output of 1.0 W were used. The wound size was 1.0 mm in diameter. Thereafter, the entire amniotic sacs with fetuses were inserted back into the maternal abdomen.

In neonatal wounded group, mice were anesthetized by diethyl ether (Wako, Osaka, Japan). Argon laser irradiations were performed on the left side of the naso-labial region five or six times, then the wound sizes were 2.0 mm in diameter. The power output was 2.0 W, while the pulse duration and wave length were the same as for the fetus operation. Neonatal mice were then returned to the cage with their mother.

Wound harvest and tissue preparation

The animals were sacrificed by an overdose of pentbarbiturate at multiple time points postwounding (PW). The experimental specimens were taken at 1, 2, 3, and 4 day PW for the fetal stages and at 1, 2, 4, 7, 10, and 20 day PW for the postnatal stages. Three to four specimens were taken at each pertinent period, but only selected period samples were described in the text. The heads were fixed with 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) and decalcified with 10% ethylenediaminetetraacetic acid (EDTA), then embedded in paraffin by a standard protocol. Serial sections of 5 μm were made parallel to the horizontal plane. The sections were stained with van Gieson stain for general histology with reference to identifying the muscle tissues and collagen fibers. note that the muscle tissues were stained yellow and the collagen fibers red by this staining.

Immunohistochemistry

The immunostaining of streptavidin–biotin method using Histofine SAB kits (Nichirei, Tokyo, Japan) was accepted as previously described (Shibata *et al*, 1997). Pretreatment with testicular hyaluronidase (type I-S; Sigma, St Louis, MO, USA) and blocking the endogenous peroxidase activity were performed. Then sections were incubated with polyclonal antibodies against FN (LSL, Tokyo, Japan; 1:500 diluted with PBS) and TN (Chemicon International, Temecula, CA, USA; 1:500) for 1.5 h at room temperature. Finally, the sections were treated with 3-amino-9-ethylcarbazole (Nichirei, Tokyo, Japan) to reveal any reaction. Negative control sections were incubated with normal rabbit immunoglobulin G (IgG) instead of the primary antibodies. Sections were observed after counterstaining with haematoxylin.

Results

Macroscopic observation

Fetal wound was almost completed without a scab and scar formation in 4 day PW (Figure 1a). Meanwhile, neonatal wound healing was completed at 20 day PW, but the wound region was covered with a smooth surface skin without skin hair and anterior view showed an asymmetric profile (Figure 1b).

Histological observations and immunohistochemistry for FN and TN

Fetal wound healing

Epithelial detachment and a relatively large tissue loss extending to the muscle tissue region were seen at 1 day PW as previously described (Suzuki *et al*, 2004) (data not shown), but re-epithelialization of the skin was completed on the wounded side at d 2 PW. Subepithelial mesenchymal cells in the dermis started to migrate from the surrounding normal tissue but the wounded region

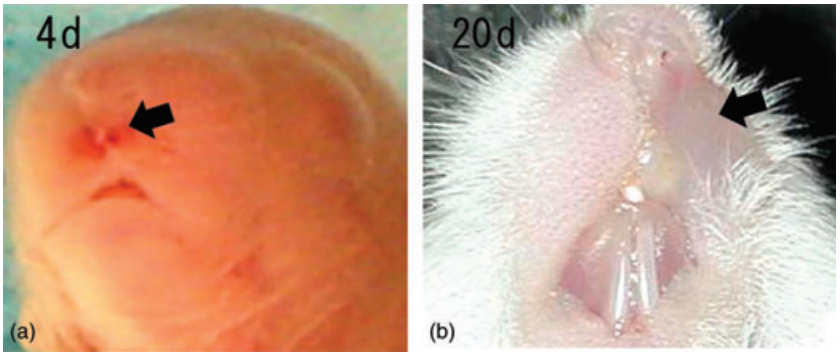

Figure 1 Macroscopic observation of laser-wound region of the fetus at 4 day PW (**a**) and neonate at 20 day PW (**b**). (**a**) Wound region (arrow) had almost healed without a scar. (**b**) Wound region was covered with smooth surface skin without hairs (arrow) and anterior view of naso-maxilla appeared asymmetry

was still identified. Muscle regeneration had started at this stage (Figure 2a).

Fibronectin immunostaining was not enhanced at 1 day PW (data not shown), but increased immunostaining was seen around the wounded region and the region in addition to diffuse immunostaining in the dermis at 2 day PW (Figure 2b). Increased TN immunostaining was also seen around the wounded region, and staining region showed a sharp distinction, compared with FN (Figure 2c).

The wounded region, containing both the epithelium and dermis was completely regenerated and dermis was filled with mesenchymal tissues at 4 day PW. However, a slight depression of the skin surface and absence of hair follicles were observed. Collagen fibers were scanty but regeneration of the muscle tissues was recognized in wounded region (Figure 2d).

The staining pattern for FN immunostaining in the wounded region at this stage was indistinguishable from the adjacent normal dermis showing diffuse immunostaining. The epidermis–dermis junction showed strong immunostaining (Figure 2e). Meanwhile, TN immunostaining was still detected around the wounded region including the epidermis–dermis junction, in the connective tissue around regenerating muscle fibers (endomysium), and in the capsules of the differentiated hair follicles (Figure 2f).

Neonatal wound healing

There was tissue loss and necrotic tissue on the wounded region at 2 day PW. A degenerative region was recognized beneath the necrotic tissue and extended into the muscle tissue region. The degenerative region was clearly distinguished from the surrounding intact tissue

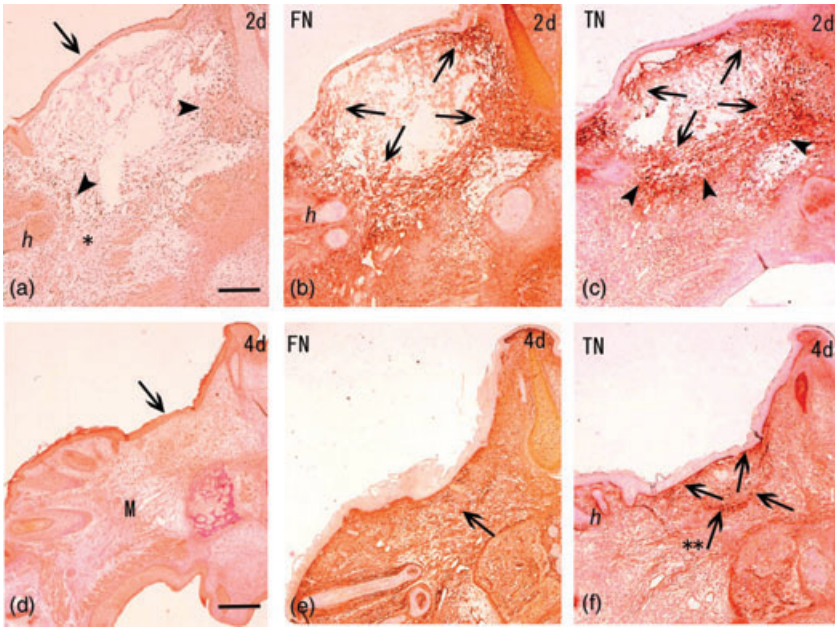

Figure 2 Histological observation of fetal wound at 2 day PW (**a**–**c**) and 4 day PW (**d**–**f**) with van Gieson staining (**a** and **d**), immunostaining for FN (**b** and **e**) and TN (**c** and **f**). (**a**) Reepithelialization of the skin was completed (arrow). Subepithelial mesenchymal tissues (arrowheads) in the dermis started to migrate from the surrounding normal tissue. Muscle regeneration had started at this stage (*). Hair follicles (*h*) had already differentiated. (**b** and **c**) Increased FN (**b**) and TN (**c**) immunostaining was seen around the wounded region (arrows). TN staining region showed a sharp distinction (arrowheads in **c**). (**d**) The wounded region was filled with mesenchymal tissues, but a slight depression of the skin surface was observed (arrow). Collagen fibers were scanty but regeneration of the muscle tissues (M) was recognized in wounded region. (**e**) The staining pattern for FN in the wounded region (arrow) was indistinguishable from the adjacent normal dermis. (**f**) TN immunostaining was detected around the wounded region (arrows), in the endomysium (**), and in the differentiated hair follicles (*h*) also showed immunostaining. Bar = 200 μm

(Figure 3a). FN immunostaining was diffusely detected in the dermis including the degenerative region beneath the necrotic tissue and in the endomysium, but not in the necrotic tissue (Figure 3b). Meanwhile, TN immunostaining was detected in the endomysium apart from the degenerative region. The degenerative region did not show TN immunostaining but capsules of hair follicles showed strong immunostaining. Furthermore, TN staining region showed a sharp distinction.

A regenerative epithelium completely covered the granulation tissue (former degenerative region) and the scab (former necrotic tissue) was almost excluded at 7 day PW. Regenerative collagen fibers filled the granulation tissue but the width of granulation tissue remained narrow. Meanwhile, muscle tissues beneath the granulation tissue were extensively increased in amount. No regeneration of the hair follicle was also seen at this stage (Figure 3d). TN immunostaining was first detected in the wounded region at d 4 PW (data not shown) and then, at 7 day PW, FN and TN immunostaining was simultaneously detected in the granulation tissue and significantly in the region just beneath the regenerated epithelium. However, the TN positive region was wider than the FN positive region (Figure 3e and f).

The wounded region was covered by a thickened and keratinized epithelium at 20 day PW. Collagen fibers increased in amount in the former granulation tissue (now recognized as regenerated dermis) beneath the epithelium, but hair follicles had not regenerated in the dermis, indicating the wounded region could now be regarded as a scar. A remarkable retraction, however,

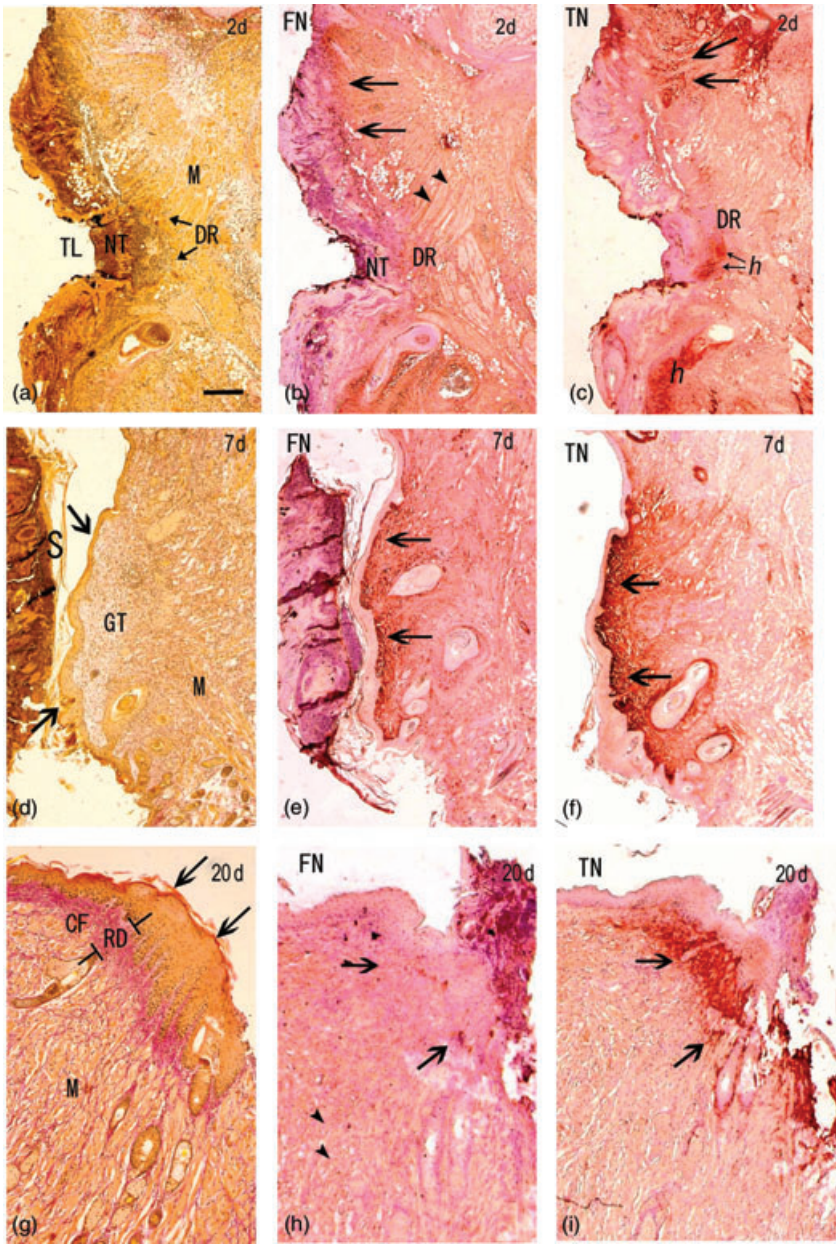

Figure 3 Histological observation of neonatal wound at 2 day PW (**a**–**c**), 7 d PW (**d**–**f**), and 20 day PW (**g**–**i**) with van Gieson staining (**a**, **d**, and **g**), immunostaining for FN (**b**, **e**, and **h**) and TN (**c**, **f**, and **i**). (**a**) Necrotic tissue (NT) was seen beneath tissue loss (TL). A degenerative region (DR) was recognized beneath the necrotic tissue and extended into the muscle tissue region (M). (**b**) FN immunostaining was diffusely detected in the dermis (arrows) including the degenerative region (DR) and in the endomysium (arrowheads), but not in the necrotic tissue (NT). (**c**) TN immunostaining was detected in the capsules of hair follicles (*h*), and in the endomysium (arrows) apart from the degenerative region (DR) that showed no immunostaining. (**d**) A regenerative epithelium (arrows) completely covered the granulation tissue (GT) and the scab (S) was almost excluded. Muscle tissues (M) beneath the granulation tissue were extensively increased in amount. (**e** and **f**). FN (**e**) and TN (**f**) immunostaining was simultaneously detected in the granulation tissue (arrows), but the TN positive region was wider. (**g**) The wounded region was covered by a thickened and keratinized epithelium (arrows). Collagen fibers (CF) increased in amount in the regenerated dermis (RD). The width of the regenerative dermis, however, was narrow, while muscle tissue (M) significantly proliferated strongly. (**h** and **i**) FN immunostaining was diffusely detected in the regenerated dermis (arrows in **h**) and endomysium (arrowheads in **h**) but significant immunostaining was not detected in the region beneath the epithelium. Meanwhile strong TN immunostaining was still detected in the regenerated dermis (arrows). Bar = 200 μm

was seen on the wounded side. Furthermore, while the width of the regenerative dermis was narrow, muscle tissue significantly proliferated strongly at this stage (Figure 3g). FN immunostaining was diffusely detected in the regenerated dermis and endomysium but significant immunostaining was not detected in the regenerated dermis (Figure 3h). Meanwhile, strong TN immunostaining was still detected in regenerated dermis (Figure 3i). Negative control sections never showed positive immunostaining at any sections examined (data not shown).

Discussion

In healing processes of the burn wound in the naso-labial region, relatively large damage remained after wound healing completed even in scarless fetal wound healing in present study.

Many cleft lip repair studies have been done in postnatal rabbits (Bardach *et al*, 1980), fetal rabbits (Longaker *et al*, 1990a; Dodson *et al*, 1991; Stern *et al*, 1992), and fetal sheep (Stern *et al*, 1993). These sequential studies also indicated the specificity of lip wound in terms of the difficulty for healing.

In general, once the wound is filled with granulation tissue and covered with the neoepidermis, the fibroblasts transform into myofibroblasts, which contract the wound (Desmoulière and Gabbiani, 1995).

In present study, a slight depression in the skin surface in conjunction with muscle regeneration was recognized at 4 day PW in the fetus. This result implies the involvement of muscle tissue in depression of wounded side. In the neonate, muscle tissues were so extensively regenerated that the width of granulation tissue and regenerated dermis remained narrow and a remarkable retraction of wounded side in conjunction with an asymmetric profile was observed at 20 d PW. Therefore, we speculate that regenerated muscle tissues instead of myofibroblasts contracted the wound and subsequently lead to the asymmetric profile. Longaker *et al* (1990a) hypothesized that creating an intact oral muscular sphincter would provide a normal functional matrix for the growing fetus. Bardach *et al* (1980) considered that lip pressure, closely related to muscle tissue, caused by the repairing wound is the main reason for the midface growth inhibition after operation. Furthermore, orbicularis oris muscle reconstruction has applied in cosmetic surgery, for example, lifting of upper lip (Santanche and Bonarrigo, 2003). These results confirm our hypothesis that muscle tissue in this region may have influences on the wound healing processes, and inhibition of muscle regeneration may reduce postoperative damages.

Fibronectin and TN are closely involved in wound healing (Mackie *et al*, 1988; Lotz *et al*, 1989; Whitby and Ferguson, 1991; Whitby *et al*, 1991; Kanno and Fukuda, 1994). During wound healing, FN has many potential roles, i.e. FN acts as a substratum for cell migration, as an opsonin and as a provisional matrix for ECM assembly (McCallion and Ferguson, 1996). When the tissue was injured, plasma-derived FN is detected in the fibrin clot 1 h after wounding in the fetal, neonatal, and adult wounds (Mackie *et al*, 1988; Whitby and Ferguson, 1991). However, in present study, FN immunostaining was but not enhanced, when compared with surrounding tissue, in the wounded region at the early stage in both types of wound healing. This is probably because of hemostasis made by the laser irradiation. FN immunostaining in the wounded region temporally increased but rapidly became indistinguishable from normal tissues. FN detected in the later stage of wound healing is produced by cells in the wound sites (Mackie *et al*, 1988; Whitby and Ferguson, 1991), and the spatial and temporal expression pattern for FN in present study is basically consistent with previous studies (Mackie *et al*, 1988; Whitby and Ferguson, 1991; Whitby *et al*, 1991; Kanno and Fukuda, 1994). FN immunostaining, however, was also detected in the endomysium, indicating this molecule is related to muscle regeneration.

Tenascin immunostaining in the wounded region was first detected at 2 day PW in the fetus and 4 day PW in the neonate. Then immunostaining was continuously detected in the wounded region, especially in the region just beneath the regenerated epithelium until the later stage of wound healing in both types of wound healing. This temporal and spatial distribution pattern of TN was basically consistent with that of previous studies (Mackie *et al*, 1988; Whitby and Ferguson, 1991; Luomanen and Virtanen, 1993) except for the delayed initial expression in the neonate probably affected by the hemostasis.

Tenascin expression in the normal adult tissue is significantly restricted, but during wound healing, a high transient expression of TN has been found coincident with the actively migrating or proliferating cells (Aukhil *et al*, 1996). TN also interacts with FN in wound healing process (Kanno and Fukuda, 1994). Although the TN expression generally disappeared in the scar after the wound contraction was complete (Mackie *et al*, 1988), it remained in the hypertrophic scar (Andriessen *et al*, 1998), keloid (Dalkowski *et al*, 1999), indicating that a continuous TN expression reflects the severity of the wound. In present study, remaining TN immunostaining at 20 day PW in the neonate indicated that a burn wound in this region is severe and may require a long time to completely heal.

Furthermore, TN immunostaining was also detected in the endomysium of the regenerating muscle tissues, although staining pattern was different from that of FN. In the normal tissue endomysium, perimysium and epimysium, no TN expression appeared (Järvinen *et al*, 2000), and no previous studies described the TN immunostaining of the endomysium during the wound healing process. Furthermore, as TN immunostaining showed a sharp distinction, it was more restrictedly expressed than FN. Recently, Wallner *et al* (2004) reported that TN is proapoptotic for muscle cells. These results indicate that expression of TN cooperating with FN seems to be important for regeneration of muscle tissues.

In conclusion, importance of muscle regeneration in conjunction with FN and TN expression in endomysium was confirmed in wound healing process both in fetus and neonate, and regulation of both molecules seems to be important to inhibit the muscle regeneration and to obtain subsequent good wound healing.

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