Ectopic expression of tenascin-C

We read, with much interest, the recent *Research Article* by Järvinen et al. (Järvinen et al., 2003), in which the authors show that, after removing the cast from an immobilized rat hind leg, within 8 weeks tenascin-C (TN-C) expression is increased in tendon and the myotendinous junction, but there is no de novo appearance of TN-C in skeletal muscle connective tissue. The authors extensively criticize our earlier paper (Flück et al., 2000), in which we concluded that loading of the chicken ALD muscle was sufficient to induce rapid ectopic TN-C expression in endomysial fibroblasts of skeletal muscle. Järvinen at al. argue that our results are merely caused by a response to muscle injury and the subsequent inflammatory reaction.

We would like to respond to these statements because the experiment by Järvinen et al. is so vastly different from ours that the conclusion, 'Mechanical loading... does not induce de novo synthesis in the skeletal muscle', as stated in the title of their paper, is not justified. We investigated the tissue 4 hours after applying an acute load to a healthy muscle; Järvinen et al. looked only at a single time point 8 weeks after reloading an immobilized (atrophied) muscle. It is possible that an acute burst of TN-C expression occurs over 8 weeks after remobilization (Flück et al., 2003). Moreover, the image of the muscle tissue hybridized with the TN-C probe in the Järvinen et al. paper (Fig. 4) is taken at low magnification (5×), and thus mRNA signals in single endomysial fibroblasts are not visible.

In our paper, we did not dismiss the possibility that the ectopic TN-C expression we observed was at least in part due to an inflammatory reaction. Nevertheless, Järvinen et al. clearly misquote us when they state in their Discussion, 'However, it is worth noting that Flück et al. (Flück et al., 2000) reported an almost 50% increase in the mass of the loaded muscle as soon as 4 hours after the beginning of elongation, concomitant with a marked early infiltration of inflammatory cells (macrophages and neutrophils) and widening of the endomysial spaces in the loaded muscle.' In fact, although we duly reported the evidence for early edema, we also demonstrated that TN-C expression after 4 hours of loading did not correlate with macrophage invasion, which we observed (by means of a specific antibody) only after 24 hours.

Although there is no doubt that the supraphysiological loading regimen applied to ALD muscle could cause immune cell infiltration and edema, and that damaged muscle fibers can induce ectopic endomysial TN-C expression, as we recently showed in a rat model for muscle loading (Flück et al., 2003), we did not observe in the chick model obvious (macro- and microscopic) signs of tissue injury or hemorrhage. In fact, reloading of rat soleus muscle caused ectopic TN-C expression occurring in a patchy manner before signs of muscle fiber injury (central nuclei) were present.

We can only repeat our arguments that mechanical stress is (at least to an appreciable extent) directly responsible for the ectopic expression of TN-C in the endomysium of loaded chick ALD muscle. TN-C mRNA expression (1) appears very early (much earlier than anybody else has ever looked so far); (2) is homogeneous throughout the muscle, not patchy as one would expect in the case of microinjuries; (3) appears before macrophage invasion; and (4) is reversible after removal of the load.

In essence, we think that the conclusions drawn be Järvinen et al. on our paper – although plausible – are not supported by their results and are driven by the inherent bias that TN-C expression must necessarily relate to muscle damage, and by the premise that the effect of mechanical loading can be reduced to the casual result of (micro)injury (Gullberg et al., 1998).

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Reply

We appreciate the keen interest that Chiquet and Flück have shown in our article (Järvinen et al., 2003). It is good to discuss the differences in our views in public, since then other researchers may become aware of some important facts and even participate in the discussion. In our case, the differences may actually arise from the apparent confusion regarding the definition and detection of skeletal muscle injury, rather than the phenomenon itself. This view is supported by the recent paper by Flück and Chiquet themselves (Flück et al., 2003), to which they also refer in their letter, a study that is in complete agreement with our findings [i.e. tenascin-C (TN-C) is induced in the skeletal muscle in response to injury]. Accordingly, we will not comment on their letter further regarding this particular issue, as there appears to be a mutual understanding between us. However, a brief comment is clearly warranted regarding their view, based on findings using the chicken wing loading model (Flück et al., 2000), that mechanical loading alone can induce de novo expression of TN-C in the skeletal muscle.

First, Flück and Chiquet have clearly not understood the experimental design of our study correctly (Järvinen et al., 2003). By stating in their letter the following, 'Järvinen et al. looked only at a single time point 8 weeks after reloading of an immobilized (atrophied) muscle. It is possible that an acute burst of TN-C occurs over 8 weeks after remobilisation', Chiquet and Flück have

not paid attention to the fact that we trained our animals twice a day during the entire 8-week remobilisation period. Accordingly, the animals we studied had been subject to two strenuous training sessions on the treadmill within the last 24 hours before they were sacrificed. Thus, the 'acute burst of TN-C' they speculated to occur in the skeletal muscle in response to mechanical loading should have also become evident in our study if mechanical loading was indeed able to induce the production of TN-C.

Furthermore, inspired by the concerns of Chiquet and Flück, we decided to perform additional corroborative experiments on a different set of specimens. Specifically, we carried out TN-C mRNA in situ hybridization analysis on specimens from another experiment (our unpublished data), in which a standardized severe contusion injury was induced in a rat gastrocnemius muscle by a strike with a spring-loaded hammer; the muscle was subsequently subjected to either non-loading (immobilization in a cast) or increased loading (treadmill training). As can be readily seen from the autoradiograph film of the mRNA in situ hybridization experiment (Fig. 1A), only a very faint expression of TN-C was found in the wound tissue in the immobilized muscle and there is no signal in the healthy parts of the muscle. By contrast, the traumatized muscle subjected to increased loading (treadmill training) showed a clear increase in the expression of TN-C in the wound tissue but, more importantly, no signal could be detected in the healthy parts of the skeletal muscle (Fig. 1B). This additional experiment clearly corroborates the findings of our previous study and confirms that mechanical loading indeed regulates the normal expression of TN-C in specific parts of the musculoskeletal tissues but cannot alone (without accompanying injury that disrupts the integrity of the tissue) induce de novo synthesis of TN-C in the skeletal muscle.

We now turn to the apparent discrepancy of how muscle injury is actually defined and detected. The authors state that '…TN-C expression after 4 hours of loading did not correlate with macrophage invasion, which we observed (by means of a specific antibody) only after 24 hours', presumably trying to suggest that this would somehow prove that no injury had occurred in response to training. However, the absence of macrophages at 4 hours does not provide evidence that TN-C expression could not be attributable to muscle damage. Rather, one should recall that in healthy muscle (tissue) there should be no inflammatory cells (neither monocytes nor macrophages) at all. Accordingly, the existence of inflammatory cells alone is definite proof that muscle damage occurred during the loading of the chicken wing muscle. As for the timing of the signs of inflammation, the authors do not take into consideration that there is always a delay in the tissue reaction to injury. The inability to detect macrophages by a specific antibody during the first 24 hours after loading (trauma) is in perfect agreement with the basic principles of acute wound healing: local injury initially results in tissue edema and exudation of leukocytes (first neutrophils, followed by monocytes) induced to emigrate across the endothelium by chemotactic agents released as a result of tissue trauma. The monocytes, in turn, differentiate into macrophages within 24 to 48 hours after initial trauma, at which point they can be identified by specific antibodies, as also shown by Flück et al. (Flück et al., 2000). Furthermore, in the classic study on segmental necrosis in skeletal muscle induced by micropuncture, Carpenter and Karpati did not observe any macrophages (identified by morphology alone and so included monocytes) until about 8 hours after the puncture (Carpenter and Karpati, 1989).

With regards to the statement '…we did not observe… obvious (macro- and microscopic) signs of tissue injury or haemorrhage after 4 hours of loading', this brings up the major problem of early detection of injury. It would be interesting to stain the chicken muscle samples at 4 hours or even earlier after the loading for either intracellular calcium or a sarcolemmal protein desmin, which might show the early injury in myofibers, or extravasated plasma proteins to show the vascular leakage. Most importantly, the authors did not explicitly provide any plausible explanation for the massive and sudden increase in muscle mass that was reported in their experiment. Such a dramatic 50% increase in muscle mass within 4 hours of loading cannot be attributable to anything but muscle trauma and the resulting inflammatory reaction. In the absence of hemorrhage, it was most likely due to the edema that the authors observed and reported – no other plausible explanation exists.

With regards to their statement 'In fact, also reloading of rat soleus muscle caused ectopic tenascin-C expression after one day occurring in a patchy manner before signs of muscle fiber injury (central nuclei) were present', it should be pointed out that central nuclei are actually a late manifestation of muscle injury, not an acute response to muscle trauma. After contusion/laceration injury some nuclei become centralized, but this takes place relatively late in the regeneration process, usually when the regenerating myotubes start to fuse with the surviving parts of myofibers.

Thus, on the basis of the facts presented above, loading of the chicken wing

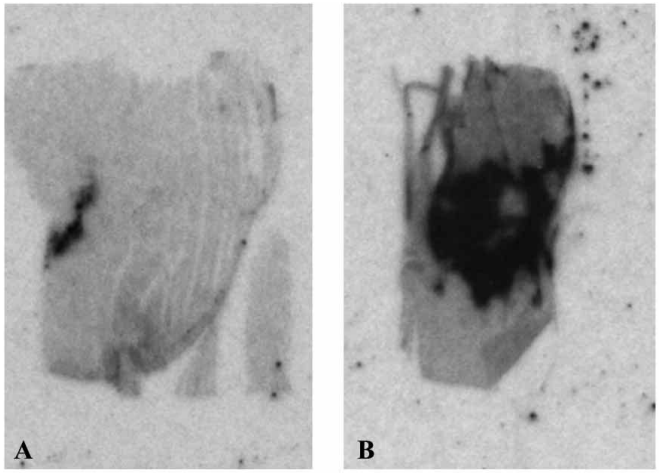

Fig. 1. TN-C mRNA in situ hybridization analysis in injured rat skeletal muscle treated either by (A) cast-immobilization or (B) treadmill running. See text for details.

obviously had caused some damage that was not detected by the methods used by Flück et al. (Flück et al., 2000), but became evident later (e.g. by the invasion by macrophages). We want to re-emphasize that our results showing that TN-C expression is induced by trauma in muscle tissue are in perfect agreement with many previous studies, including the recent study by Flück and Chiquet themselves (Flück et al., 2003). Accordingly, we believe that the conclusions in our criticized study (Järvinen et al., 2003), which claims that mechanical loading regulates the normal expression of TN-C in the musculoskeletal tissues, but disruption of the mechanical integrity of the tissue is required for induction of de novo synthesis of this protein, are correct. It would be most interesting to clarify by which mechanism the injured myofibers induce the early production of TN-C mRNA in endomysial fibroblasts in the chicken wing loading model. Solving this problem requires such wide expertise in molecular biology as Flück and Chiquet undeniably have.

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Rebuttal

We would like to thank Järvinen et al. for their extensive and thoughtful reply to our letter. We basically agree with many of their arguments. We are especially interested in their new data indicating a synergy between (micro)injury-induced inflammation and mechanical stress in inducing tenascin-C (TN-C) expression. This is where our different views might meet: mechanical stress is perhaps not sufficient, but nevertheless essential, for TN-C induction both at normal and ectopic sites.

Järvinen et al. provide autoradiographs of in situ hybridizations to document their new set of experiments. Unfortunately, as is the case in their former paper (Järvinen et al., 2003), these photographs are taken at very low magnification (5-10×). Under these conditions it is not possible to determine the cellular source of the hybridization signal. Moreover, if there was low to medium level expression of TN-C mRNA in endomysial fibroblasts (e.g. in uninjured but loaded parts of the muscle) it remained undetected. If we photographed our old samples of loaded chick muscle at a magnification this low we would barely see the signal, although its cellular specificity becomes very obvious at 40-300× (see Flück et al., 2000). Nevertheless, the synergy hypothesis by Järvinen et al. is indeed worth being tested further. Concerning the points raised regarding the experimental design, the high magnitude of mechanical factors acting on the muscle during the predominately eccentric, low-repetitive (unidirectional) ALD muscle stretch (Flück et al., 2000) compared with that acting on the muscle during the concentric, high-repetitive contractions with running on the inclined treadmill (Järvinen et al., 2003) may explain part of the difference in TN-C mRNA expression in the two reports (Lindstedt et al., 2001).

Lastly, Järvinen et al. seem to think that TN-C induction in muscle fibroblasts must involve a paracrine factor originating from the injured and/or loaded muscle fibers, and urge us to test this. In cultured fibroblasts, however, we have demonstrated that tensile stress can directly induce TN-C in the absence of other cells (for a review, see Chiquet et al., 2003); the same has been shown for heart myocytes (Yamamoto et al., 1999). Medium conditioned by stressed cells does not induce TN-C in resting fibroblasts. In our hands, serum and certain growth factors such as TGF-β act in an additive (rather than a synergistic) way with tensile stress to induce TN-C expression. These and other results obtained in vitro should be taken into account when designing experiments in a more complex in vivo environment.

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