Interferon Inhibits Myogenesis *In Vitro* and *In Vivo*

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Abstract

Interferon (IFN) is an endogenously produced protein that has been implicated in the regulation of a number of different cellular processes. Included in the cellular systems studied to date is the skeletal muscle lineage, which provides a useful model for the study of differentiation as this process is clearly marked by the fusion of mononuclear cells into bi and multinucleate myotubes. The effect of IFN on the differentiation of muscle cells has however been disputed in the literature. The experiments reported here establish that IFN has an inhibitory effect on the differentiation of cultured muscle cells by delaying the fusion of mononuclear cells into myotubes in a dose dependent fashion. This was observed in the C_2C_{12} muscle cell line and in primary muscle cultures from Swiss SJL/J and BALB/c mice. When IFN was injected directly into the muscles of mice of these strains, SJL/J mice showed minimal damage in contrast to BALB/c mice where significant damage was observed. Administration of IFN to regenerating muscle of adult SJL/J mice, where normally quiescent muscle precursor cells have become activated, demonstrated persistent necrotic tissue and increased fibrosis in addition to reduced myotube formation and provides further evidence for an inhibitory role of IFN in muscle differentiation.

Key words: skeletal muscle, interferon, myogenesis, differentiation, regeneration, *in vitro, in vivo.*

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The IFN family of proteins have long been known for their antiviral, antiproliferative and immunomodulatory effects on cells (reviewed in 22). In addition, IFNs are becoming increasingly recognised as regulators of a variety of cellular events including cell growth, proliferation, differentiation and expression of specific genes. IFNs are known to be implicated in regulation of proliferation and differentiation in a number of cell types [2, 16, 19], and the relevance of this to skeletal muscle is of particular interest. Cultured cells of the skeletal muscle lineage have been extensively studied because they provide a unique system for investigating the morphological and biochemical features of differentiation. Precursor cells of muscle undergo repeated cycles of cell replication to expand the population of cells, which then withdraw from the cell cycle and undergo differentiation. This differentiation process is detectable morphologically by the fusion of mononuclear cells into multinucleate myotubes with contractile ability. This process is also accompanied by numerous biochemical changes including expression and synthesis of contractile proteins, appearance of acetylcholine receptors, an increase in the activity of enzymes such as creatine kinase and acetyl cholinesterase, a reduction in DNA polymerase activity and reduced DNA synthesis. Skeletal muscle cells have been examined for the effects of IFN on both cellular proliferation and differentiation, however some of the results have been conflicting.

Using primary cultures of chicken myoblasts, Lough et al. [17] demonstrated inhibition of myogenesis using a preparation of chicken IFN with low specific activity, which acted by blocking differentiation. In addition, a commercially available IFN preparation of high specific activity was examined on the near tetraploid rat muscle cell line MM14DZ, and a similar effect was observed where creatine kinase levels were reduced and myotube formation blocked [20]. In contrast, Fisher et al. [5] demonstrated an acceleration in myotube formation and enhanced creatine kinase activity following treatment of primary human

myoblast cultures with both crude and recombinant IFN preparations. These papers highlight discrepancies in the literature with respect to the effect of IFN on the differentiation of skeletal muscle cells. To this date there has been little work done to resolve this issue.

The purpose of this study was to determine the *in vitro* effect of IFN on muscle cells in an attempt to resolve the conflict in the present literature. Having determined the effect of IFN on cultured cells, the IFN effect on myogenesis *in vivo* was investigated in skeletal muscle of mature mice regenerating after crush injury [8]. Skeletal muscle of both Swiss SJL/J and BALB/c mice was examined as it has been shown previously that new muscle formation is more effective in both minced graft and crush injured muscle of the SJL/J strain [8,9,18]. This is associated with the earlier onset of muscle precursor cell replication [8], much greater cellularity and enhanced myotube formation [18] in SJL/J compared to BALB/c mice. Because of the consistent difference in the regenerative response it was of interest to compare the effect of IFN in these two strains.

Materials and Methods

Animals

Inbred female Swiss SJL/J and BALB/c mice aged 6-8wks obtained from the Animal Resource Centre, Murdoch, Western Australia were used according to the guidelines and handling procedures outlined by the National Health and Medical Research Council of Australia.

Tissue culture experiments

Primary cultures of muscle precursor cells were prepared by enzymatic digestion of skeletal muscle removed from the hind limbs of adult (6-8 week old) BALB/c and SJL/J mice using collagenase and trypsin. Cells were cultured in Dulbecco's modified Minimal Essential Medium (DMEM) containing 10% horse serum (Cytosystems, Australia), 40μg/mL dexamethazone (David Bull Labs), 1μg/mL linoleic acid (Sigma), and 30μg/mL gentamicin (Delta West, Australia). The myogenic cell line C_2C_{12} (obtained from American Type Culture Collection ATCC), derived from the C3H mouse strain, was grown in DMEM with 10% horse serum and 30μg/mL gentamicin. All cells were plated at approximately $5x10^3$ cells/35mm diameter well and one day after seeding the primary cultures were washed with fresh medium to remove cell debris. On the second day after seeding the medium of all cultures was replaced with fresh medium containing either 20, 200 or 2000 IU/mL of mouse IFN-α/ß ($1.7x10^6$ IU/mL, specific activity $5.0x10^6$ IU/mg: Lee Biomolecular #20061). Control cultures received the same medium without IFN. The medium with the appropriate IFN concentration was replaced every 48hr and respective fields were photographed daily to compare the extent of cell fusion.

In vivo experiments

The effect of IFN on normal skeletal muscle was assessed by injecting IFN-α (concentration of $4.5x10^5$ IU/mL, specific activity of $5.5x10^5$ IU/mg: Lee Biomolecular #22051) in a 5μL volume ($2.25x10^3$ IU/dose) directly into the right TA muscle of BALB/c and SJL/J mice daily for either 1, 4 or 9 days. The left tibialis anterior (TA) muscles of the same mice were injected with buffer (0.4M Glycine HCl buffer, pH 3.5) for similar times to serve as a non-IFN injection control. Injections were performed using a Hamilton syringe with a 33 gauge needle, where the needle was passed through the skin covering the TA muscle and inserted into the muscle parallel to the orientation of the fibres with the needle pointing toward the foot. All animals were sacrificed on the 9th day after the initial injection.

The effect of IFN on regenerating adult muscle was assessed in adult, female SJL/J mice following crush injury to the midregion of the TA muscle [8]. IFN or buffer was injected into the area of muscle damage at the time of injury and on subsequent days to give a total of either 4 or 9 injections. Animals were sacrificed on the 9th day post injury and the TA muscles removed and processed for histology. Mice that received a crush injury but no injections were included as additional controls to assess the extent of damage caused by the daily injections.

Results

Effect of interferon on cultured muscle precursor cells

IFN treated primary muscle cultures from both BALB/c and SJL/J mice showed a dose dependent inhibition of myotube formation. Although fusion was inhibited by exposure to IFN it did not appear to affect proliferation of the muscle precursor cells in either strain as all cultures were at the same density prior to fusion occurring in the various cultures (data not shown). With the highest IFN concentration (2000 IU/mL) myotube formation was inhibited compared to control cultures, particularly at the early times following IFN exposure. In non-IFN treated control cultures, fusion was observed as early as 2 days following the time of IFN addition (equivalent to 4 days post cell seeding), where small numbers of binucleate cells were present. Similar binucleate cells were observed at 2 to 3 days after treatment with 20 and 200 IU/mL IFN and later, around 3 to 4 days, in cultures treated with 2000 IU/mL. Cells appeared fusiform and elongated in shape and demonstrated close alignment with adjacent cells, however fusion of these cells was delayed in cultures treated with IFN, particularly at the higher concentration of 2000 IU/mL. By 6 to 7 days post-IFN treatment in cultures of SJL/J muscle the degree of myotube formation had essentially caught up with that in the other IFN treated and control wells (Figure 1a-d). In contrast, cultures of muscle from BALB/c mice at the same stage showed fewer myotubes in IFN treated compared to control non-treated cultures (Figure 1e-h).

To compare results from these primary muscle cultures with that of a muscle cell line, C_2C_{12} cells were examined in a similar manner. As before there was no apparent effect

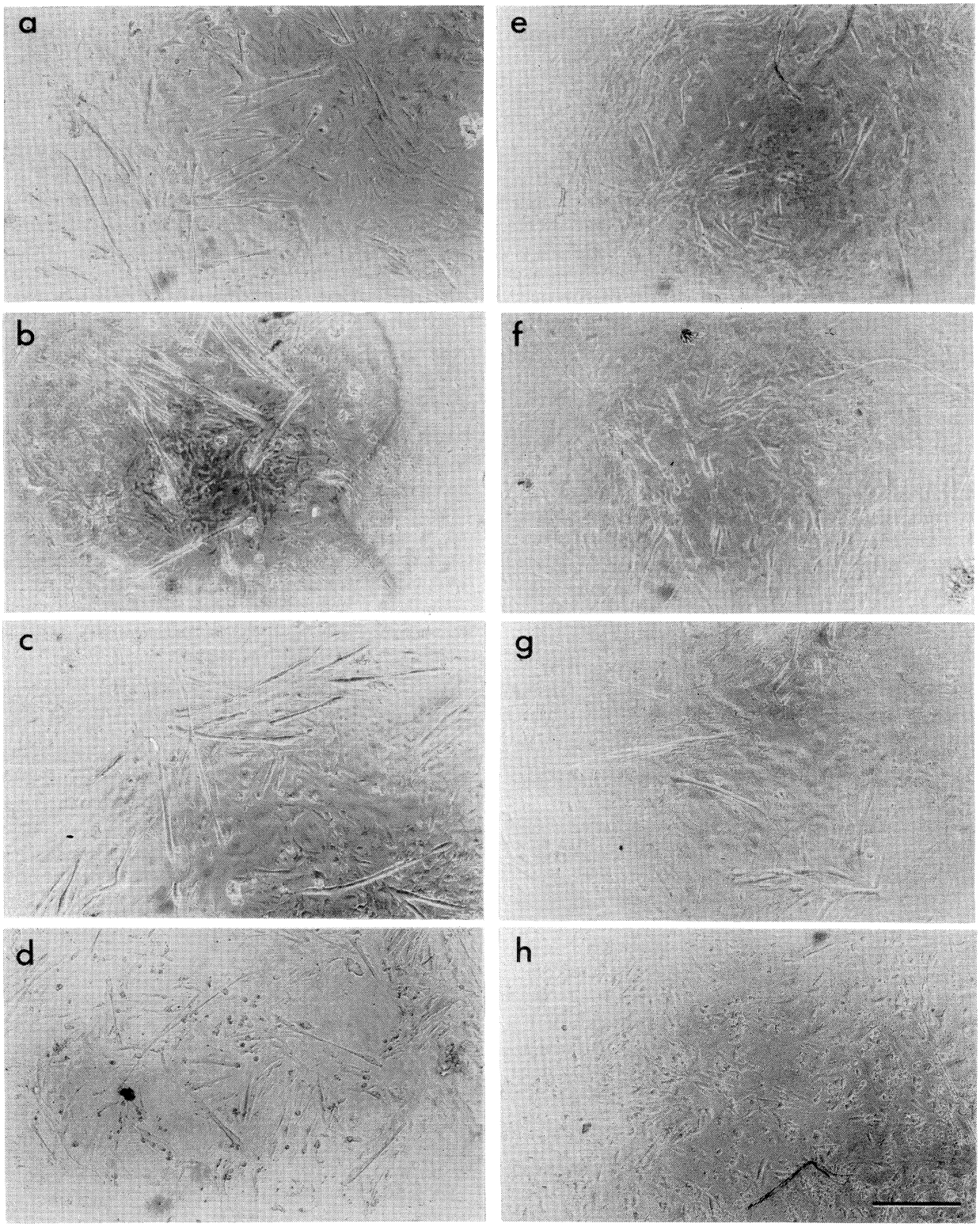

Figure 1: Primary cultures from SJL/J muscle at 6 days post IFN exposure (a,d) and BALB/c muscle at 7 days post IFN exposure (e,h) with no IFN (a,e), 20 IU/mL (b,f), 200 IU/mL (c,g) and 2000 IU/mL (d,h) of IFN-α/ß Myotubes were well formed in muscle from SJL/J mice at all IFN concentrations whereas myotubes were less conspicuous in cultures of BALB/c muscle exposed to 2000 IU/mL IFN (h). Bar = 150 μm

on cell proliferation and, in contrast to the primary cultures, these cells appeared to become fully confluent prior to fusion. Evidence of fusion in control cultures was observed at about 5 days following the initial IFN exposure (equivalent to 7 days post cell seeding) and was also observed at the same time in cultures treated with 20 IU/mL IFN. In cultures treated with 200 IU/mL IFN fusion was not observed until day 6 post-IFN exposure and at day 9 for cultures treated with 2000 IU/mL (data not shown). The myotubes in the latter cultures remained smaller than those in control and those treated with 20 and 200 IU/mL until about day 15 post exposure when all cultures were indistinguishable.

These experiments demonstrate that IFN acts on both primary muscle cultures and a myogenic cell line by transiently preventing fusion into multinucleate myotubes. In muscle cells from SJL/J mice this inhibition was overcome at about 6 to 7 days post-IFN treatment at which stage test and control cultures appeared indistinguishable. However, in cultures of muscle from BALB/c mice, differences in the amount of fusion were still evident at this time, and test and control cultures became indistinguishable 2 to 3 days later. In the C_2C_{12} cell line a similar delay of fusion was noted with the higher IFN concentrations, although a longer period of time was required for test and control cultures to appear equivalent. These observations support the previous findings that differentiation in both primary muscle cultures and skeletal muscle cell lines was inhibited by IFN [17, 20], and contrasts with the data of Fisher et al. [5]. Having established the effect of IFN to be inhibition rather than enhancement of myogenesis, further experiments were undertaken to determine whether a similar effect could be demonstrated in muscle *in vivo*.

Effect of interferon on normal uninjured skeletal muscle in vivo

Muscle of normal uninjured BALB/c and SJL/J mice which received a single intramuscular injection of IFN showed virtually no histological indicators of muscle damage when analysed 9 days later. Both strains of mice showed a small number of basophilic fibres with central nuclei and some infiltrating cells, however these were localised to a small area of the muscle and were also observed with buffer treated specimens, consistent with damage from the trauma of injection (data not shown).

In SJL/J mice injected for 4 or 9 days there appeared to be little difference between the histology of IFN and buffer injected muscles. A small amount of muscle damage was observed including some basophilic fibres showing central nucleation in the presence of inflammatory cells in both buffer and IFN injected samples. After 9 daily injections there appeared to be slightly more damage in both IFN and control samples when compared to only 4 daily injections, consistent with the increased number of injections. Although there were no significant differences between IFN and control treated samples with SJL/J mice (Figure 2a and b), a slight swelling of the fibres in IFN treated muscles was noted.

BALB/c mice injected with buffer (Figure 2c) showed similar histological features to SJL/J mice which had been injected with either buffer or IFN (Figure 2a and b). In contrast, injection of BALB/c muscle with IFN (Figure 2d) produced a striking effect. Daily injections of BALB/c muscle with IFN for 4 or 9 days resulted in substantial disruption to the muscle tissue, characterised by numerous necrotic fibres and infiltration of inflammatory cells. Many necrotic fibres were calcified and numerous surviving fibres displayed disruption and central nucleation. In addition there was significant fibrosis around the region of injection (Figure 2d). IFN treatment of BALB/c muscle thus displayed significant deleterious effects when injected directly into normal adult skeletal muscle. Due to the significant damage to muscle of BALB/c mice in response to IFN injection, SJL/J mice were used for subsequent experiments to examine the *in vivo* effect of IFN on regenerating adult muscle.

Effect of interferon on myogenesis in vivo

The regime of IFN and buffer injections was repeated in muscles of SJL/J mice regenerating after muscle injury to examine the *in vivo* effect of IFN on active myogenesis in mature muscle. IFN or buffer was injected at the site of crush injury immediately following injury and on subsequent days to give a total of either 4 or 9 injections, and muscles sampled on the 9th day post-injury. Macroscopic examination of the muscles prior to dissection indicated that healing of the injured muscle was impaired in the IFN treated samples. A thickening of the muscle fascia over the area of injury in buffer injected muscles, was similar to that seen with injured but non-injected control samples. Macroscopically the lesion in injured, buffer-injected samples appeared indistinguishable from the injured, non-injected controls. Conversely, crush injured muscles injected with IFN had a raised area over the injury site indicative of oedema and fibrosis with evidence of calcification, in addition to thickening of the fascia. The dramatic macroscopic effect varied between mice but there was a marked difference between the regeneration of IFN and buffer injected muscles. These macroscopic differences were also reflected histologically.

The non-injected control muscles showed the typical picture at 9 days post crush injury where the central zone of injury contained some infiltrating inflammatory cells and basophilic staining myotubes with little or no remaining necrotic muscle tissue. The area adjacent to the crush site was highly cellular and contained numerous basophilic myotubes with central nuclei (Figure 3a). Buffer injected controls showed a similar picture to the non-injected controls, although there was greater variation between samples, most likely as a result of the increased trauma caused by the daily injections over the 9 day period (Figure 3b). The histological picture for buffer-injected regenerating muscles showed infiltrating inflammatory cells and successful myotube formation. Conversely, injection of IFN into crush injured muscle appeared to prevent effective removal of necrotic fibres as evidenced by large amounts

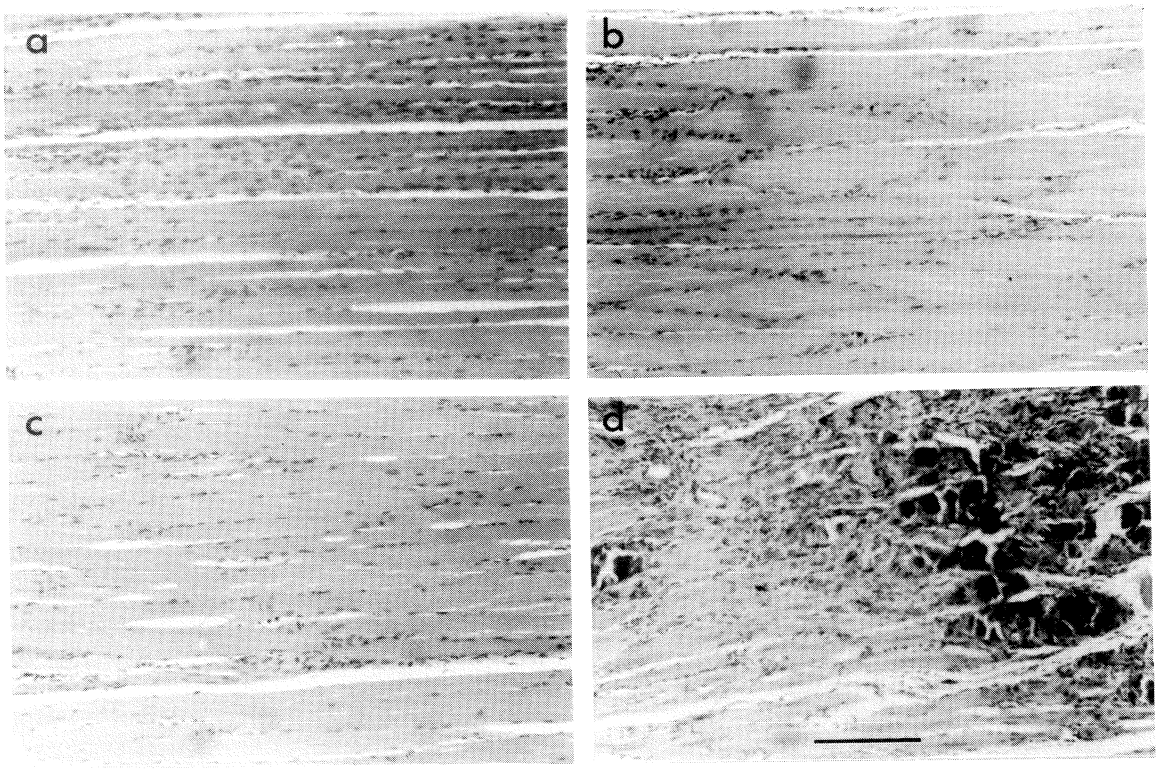

Figure 2: Haematoxylin and eosin stained sections of tibialis anterior muscle from SJL/J (a,b) and BALB/c (c,d) mice 9 days after the initial of 4 daily intramuscular injections with buffer (a,c) and IFN (b,d). There was little difference between buffer and IFN injection in SJL/J mice whereas IFN injection in BALB/c mice produced severe necrosis, calcification and cellular infiltration. Bar = x150 μm

of necrotic material in 75% of the samples analyzed, some of which were calcified (Figure 3c). There was an increase in the fibrous tissue content and fewer myotubes in IFN treated muscles.

In general, crush injured muscle injected with IFN daily for either 4 or 9 days showed reduced healing and muscle regeneration when examined at 9 days after crush injury. This was demonstrated by the persistence of necrotic debris at the site of injury, calcification of some of these necrotic fibres, an increase in fibrous tissue and fewer myotubes in the muscle. Together with the tissue culture studies, these results indicate that IFN inhibits myogenesis both in vitro and in vivo. Quantitation of the effect *in vivo* is significantly more difficult to achieve given variability in the crush injury, the injection regime and the individual animals.

Discussion

The primary effect of IFN, and indeed the reason for the initial discovery, was the antiviral property exhibited by these molecules [13]. The inhibitory effects of IFN on the proliferative capacity of cells was recognised soon after [21]. More recently, IFNs have been shown to be involved in the regulation of differentiation of cells [2, 16] in a wide range of cell types [4, 12, 14, 15, 23, 24].

Previous in vitro experiments analyzing the effect of IFN on differentiation of myoblasts identified both inhibitory [17, 20] and stimulatory [5] effects. Primary cultures were examined by all groups, yet contrasting results obtained. The effects were not due to the antiproliferative effect of IFN as cultures were at the same density prior to fusion, in agreement with the previous studies [5, 17, 20] where a slight increase was noted [20] and was due to the inhibition of differentiation which allowed the mononuclear cells to undergo further round/(s) of cell division. The tissue culture results presented in this study for primary muscle cultures of both BALB/c and SJL/J mice and the C_2C_{12} myogenic cell line, provide evidence in agreement with the inhibitory effect of IFN on skeletal muscle differentiation. The experiments describing the increased differentiation of human muscle cells in primary cultures exposed to IFN [5] thus stand in unexplained opposition to all other results. This may reflect wider differences between muscle cells of human compared to nonhuman species, since different responses have been shown for basic Fibroblast Growth Factor and Epidermal Growth Factor in muscle cells between species [10, 11].

Strain-specific differences were observed when comparing the IFN effect on primary muscle cultures from SJL/J and BALB/c mice, where the delay in fusion resulting from IFN was longer in BALB/c compared to SJL/J cultures. Strain-specific differences in the response of muscle to

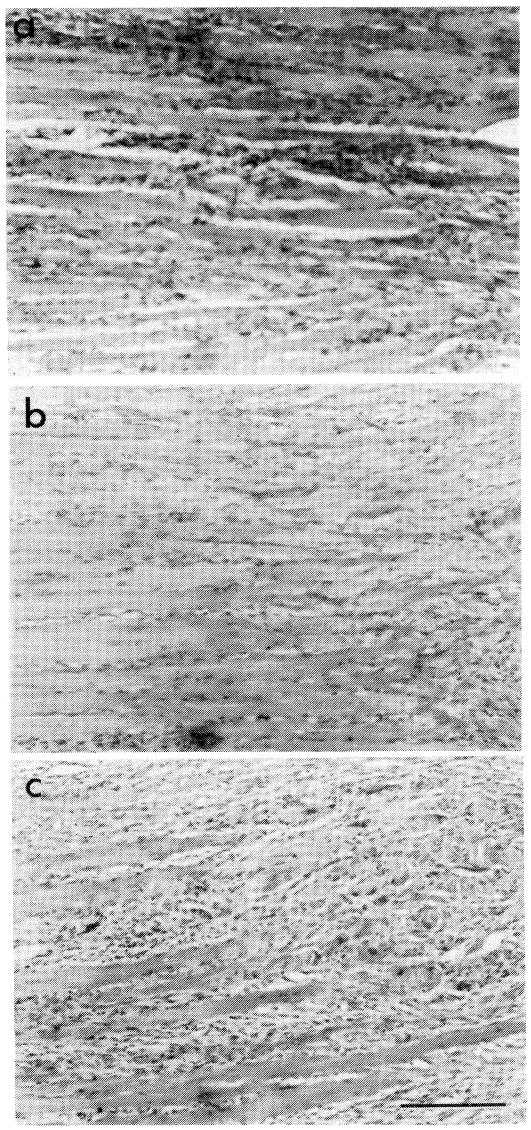

Figure 3: Haematoxylin and eosin stained sections of crush injured TA muscle at 9 days post injury with no injection (a), buffer injection (b) and IFN injection (c) at the time of injury and on 3 subsequent days. The undamaged area of muscle is toward the left and the crush injury is toward the right. IFN treatment showed increased necrotic and fibrous tissue, and some of the necrotic fibres had become calcified. Bar = 150 μm.

IFN were also noted *in vivo*. Multiple injections of IFN into normal muscle showed virtually no effect in SJL/J mice whereas injected muscles of BALB/c mice were characterised by numerous necrotic fibres (many of which were calcified), myofibres with central nuclei, many infiltrating inflammatory cells and fibrosis around the region of injection. These experiments support the observations that SJL/J mice show a different and superior response to muscle injury in comparison to BALB/c mice [8, 9, 18].

The only effect IFN appeared to have on normal muscle from SJL/J mice was a small degree of fibre swelling, whereas in BALB/c mice there was substantial fibre necrosis with subsequent calcification. Young BALB/c mice of less than 4 weeks of age are more prone to calcification of necrotic muscle than other strains [6], although this is less common in older animals. It has also been observed that intraperitoneal injections of IFN-α/ß in CDF1 mice (Sidky, YA. pers. comm.) result in ultrastructural changes and myofibre necrosis in skeletal muscle. Since CDF1 mice are a cross between DBA/2 and BALB/c strains, this deleterious effect of IFN on normal skeletal muscle may well be related to the BALB/c genotype. The exact mechanism of the increased sensitivity of BALB/c muscle to IFN remains to be elucidated.

Numerous factors participate in the myogenic process *in vivo* including inflammatory cells involved in removal of cellular debris, fibroblasts which contribute to the repair process, regulatory molecules such as polypeptide hormones produced by various cell types, serum-derived substances, the vascular supply of nutrients and the possible recruitment of myogenic cells from interstitial connective tissue (reviewed in 7). It is not possible to rule out the effects of IFN directly on these processes *in vivo* in the present experiments, however the reduction in myotube formation does point to an effect on the muscle cells themselves. In addition, the effect of IFN on injured SJL/J muscle, which was not seen with IFN treatment of uninjured SJL/J muscle, suggests an effect of IFN on the repair of the damaged muscle cells. The data presented here using the crush injury model of regeneration indicates that IFN produces an inhibitory effect on myogenesis *in vivo* and supports the *in vitro* observation in mouse muscle cultures. Together these results further substantiate the previous reports of differentiation inhibition in muscle cultures [17, 20].

These results establish an inhibitory effect of IFN both *in vitro* and *in vivo* on myogenic differentiation. Although the molecular basis for this effect remains to be defined, it is postulated that the effect may be mediated by the IFN system. This system is induced in response to IFN and is comprised of a number of enzymes, the most characterised of which are 2',5'oligoadenylate synthetase and a protein kinase, both of which are activated by double stranded RNA (reviewed in 2). These enzymes act to inhibit protein synthesis and since differentiation involves the *de novo* synthesis of numerous new proteins the IFN system may represent the appropriate mechanism to inhibit differentiation [1], although it is likely that more complex mechanisms are also involved. Interaction of IFN with cellular oncogenes is another possible mechanism, as IFN has been shown to down regulate c-myc in various cell lines [3, 15, 25]. The molecular mechanism by which IFN modulates the myogenic program is not clearly defined and it is possible that the multiple effects of IFN described by Tomita and Hasegawa [27], represent differences in IFN

concentration, time of exposure during the cell cycle and interaction with other factors.

Damage to skeletal muscle fibres by IFN is of clinical interest as IFN is administered intramuscularly to patients with carcinomas including B-cell lymphomas, T-cell leukemias such as Koposis Sarcoma, and bladder and ovarian cancers [26]. Therapeutic dosages are generally in the order of 0.5-50x10^6 IU/mL per intramuscular dose and it is commonly reported that these patients experience muscle pain. This pain may be cause by IFN damage to muscle fibres in a similar way to that demonstrated in these experiments, and the variation observed between patients is similar to the strain differences demonstrated here.

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