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Apoptosis of Rat Myoblasts is Induced *in Vitro* by Late, but not Early, Wound Fluids

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Abstract

Interstitial fluids from late wound repair environments are not "regeneration permissive" as judged by their inability to promote either proliferation or differentiation of myoblasts in vitro. This investigation considered whether apoptosis (induced death) was an alternative fate for myoblasts exposed to these interstitial fluids. Myoblast fate was assessed by cell counts, tritiated thymidine release, and propidium iodide staining. Fluids from early wounds increased mean cell counts and induced little thymidine release or propidium iodide labeling. In contrast, fluids from late wounds reduced cell counts and induced both thymidine release and propidium iodide labeling. These data suggest that interstitial fluids from late wounds might trigger apoptosis. It is presently unclear how death is induced in these environments. Further study is required to ascertain whether these effects represent an impediment to skeletal muscle regeneration only or if they reflect a previously unidentified role for late wound fluids in postinjury resolution.

Introduction

Following injury, adaptive mechanisms are initiated that culminate in repair with replacement of damaged parenchymal cells by scar tissue or regeneration with functional and, often, structural restoration of damaged tissues or organs (Goss, 1992). Typically, repair and regeneration are mutually exclusive responses to injury (Sicard, 1998). In addition, repair can be induced even in tissues that respond to injury by regenerating (Sicard, 1985). Thus, one might infer that differences exist in cellular interactions at the site of injury and that these interactions differentially modulate activities of potential effector cells. Since functional quality of a damaged tissue or organ depends upon the outcome of postinjury resolution, understanding the nature and consequences of these local interactions is of paramount importance.

One way to envision local interactions at sites of injury is as a competitive balance between putative, mutually-exclusive effector mechanisms. Specifically, such an hypothesis suggests that favoring proliferation and functional expression of stromal cells, e.g., fibroblasts, would lead to repair. Alternatively, proliferation and increased functional expression of parenchymal cells (or their precursors) favor regeneration. Understanding important distinctions between mechanisms of repair and regeneration requires identifying and characterizing factors that differentially affect responsiveness of putative effector populations, contrasting physiological events that modulate activities of effector cells, and defining strategies for arresting or reintegrating into the damaged tissues the activated effector cells.

This laboratory has been exploring influences of a mammalian repair environment, as represented by interstitial fluids recovered from polyvinyl alcohol (PVA) sponges, on rat neonatal myoblasts, as an analog for regenerating muscle, *in vitro*. The PVA sponge model mimics events of repair in normal wounds (Schilling et al., 1959; Hølund et al., 1979) and provides a convenient way to recover interstitial wound fluids (WFs) containing relevant soluble bioactive factors from wound environments. Previously, Sicard and Nguyen (1994) devised an *in vitro* assay system to test the ability of PVA sponge repair environments to support events required for skeletal muscle regeneration. For this assay, a "regeneration permissive" environment is defined as one in which myoblasts proliferation is promoted by early WFs and myoblast differentation is induced by late WFs. Accordingly, if early WFs (e.g., 1 – 7 days postimplantation) stimulate myoblast proliferation and late WFs (e.g., 10 – 15 days postimplantation) promote myoblast differentiation then the repair environment would be considered "regeneration permissive."

Using this assay, Sicard and Nguyen (1994) concluded that the PVA sponge repair environment

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could not fully support skeletal muscle regeneration. In particular, they observed that, while early WFs were mitogenic, late WFs failed to stimulate either proliferation or differentiation of rat neonatal myoblasts *in vitro*. Myoblasts in culture typically display two patterns of response. In the presence of growth factors, they actively proliferate and upon withdrawal of growth factors they differentiate (Florini et al., 1991). Since late WFs stimulate neither proliferation nor differentiation of rat L8 myoblasts in culture, might they have some other effect? If so, apoptosis, or programmed cell death, is a likely candidate (Schwartzmann and Cidlowski, 1993; McKenney, Romzek, and Ziemba, 1999). Apoptosis is known to play a significant role in development (Clark, 1990) and might represent a default state for embryonic-like cells that are neither progressing through cell cycle nor differentiating. Alternatively, our myoblasts might require paracrine or autocrine signals for survival, as suggested by Raff (1992) for cells in general, that are either absent from or blocked in this environment.

The purpose of the present investigation, therefore, was to determine effects of interstitial WF on myoblast survival *in vitro* and ascertain whether late WFs might promote apoptosis of rat myoblasts *in vitro*.

Materials and Methods

Generation of WFs

PVA sponges (Unipoint Industries, Inc., Highpoint, NC) were washed extensively in distilled water then cut into 0.5 cm^3 blocks (1 X 1 X 0.5 cm). Following an overnight wash in running water, sponge blocks were transferred to 0.9% NaCl, steam sterilized (autoclaved for 20 minutes), then stored at 5 – 8° C until used.

Sterile sponges were implanted subcutaneously onto the backs of young adult male Fischer rats (150 – 175 g; Sasco, Inc., Madison, WI) under nembutal (sodium pentobarbital; Abbott Laboratories, Chicago, IL) anesthesia. Midline incisions were made through the skin. Five sponges were implanted on each side of these incisions which were then closed with surgical clips. At specific times (1, 3, 5, 10, and 15 days) after implantation, rats were euthanized by carbon dioxide asphyxiation and sponges rapidly removed. Following decapsulation, sponges were cut and pooled in 3 mL syringes inserted into 50 mL conical tubes. WF pools for each time point were prepared by expressed fluids from these sponges through two cycles of centrifugation at 3,000 Xg for 10 minutes. WF pools were stored at –80° C until used.

Procedures involving animals were reviewed and approved by the Animal Care and Use Committee at the University of Minnesota. All procedures conformed to the guidelines established by the National Institutes of Health and promulgated in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23).

Myoblast Cell Line

Myoblasts used in this investigation were from the neonatal rat L8 cell line established by Yaffe (1968). Cells (CRL-1769) obtained from the American Type Culture Collection (ATCC, Rockville, MD) which were received in pass 21 and used between the 23rd and 28th passages. Cells were routinely maintained in medium consisting of Dulbecco's modified Eagle's medium (DMEM) and Medium 199 (4:1, v:v; base medium) supplemented with 10% calf serum and 1% chick embryo extract.

Evaluation of WF Effects

Cell Counts:

Immediate acute effects of exposure to WFs was assessed by means of cell counts. L8 cells were inoculated into 24 well plates at a density of 3,000 or 30,000 cells per well in their routine maintenance medium. The following day, medium was removed and replaced with base medium (designated as controls) or base medium supplement with 10% (v:v) WF. After 24 hours, media were removed and saved, plates were rinsed with phosphate buffered saline (PBS), and cells harvested by trypsinization. Suspensions of harvested cells were added to their respective media before centrifugation at 3,000 Xg for 5 minutes. Cell counts were performed using a hemocytometer.

A series of six wells were harvested and cell counts were performed at the time of medium replacement in order to establish the reference number of cells present following the initial overnight incubation. Cell counts for each series then were normalized against this reference number and percent recovery determined. Recoveries in excess of 100% presumably represented net cell proliferation whereas recoveries below 100% represent net cell loss that occurred during the second incubation under test conditions.

Tritiated Thymidine Release:

An index of cell death was obtained by measurement of tritiated thymidine (*TdR; methyl-[^{3}H]-thymidine, 6.7 Ci/mmol: New England Nuclear Corp., Boston, MA) release (Wright et al., 1993). Cells were inoculated as above; however, 2 µCi/well of *TdR was included during the first incubation period. Medium was removed and wells were washed three times with PBS. Challenges were initiated by adding either base medium (no serum or WF) or base medium supplemented with either calf serum or day 1, day 10, or day 15 WF. All supplements were added to achieve a final concentration (v:v) of 10%. Incubations were

resumed for up to 48 hours. The amount of *TdR released to the medium was determined following 24 or 48 hours' incubation. In addition, the amount of *TdR released by cells at the onset of the incubation period was determined.

After the designated incubation interval, *TdR release was determined as follows: (A) Medium was removed and wells were washed with PBS. Medium and wash were pooled and an aliquot counted to yield a measure of *TdR released into the medium. (B) Lysis buffer (0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris•HCl [pH 8.6]); 450 µL/well) was added to each well and plates were incubated at 4°C overnight. Lysate was harvested from each well and each well was washed with fresh lysis buffer. Lysate and wash were pooled and centrifuged at 14,000 rpm in an Eppendorf microcentrifuge (Model 5415) for 10 minutes. Supernatants were removed and the pellet washed once with 450 µL fresh lysis buffer. The original and wash supernatants were pooled and an aliquot counted to provide a measure of *TdR in fragmented DNA. (C) The final pellet was resuspended in 450 µL suspension buffer (1 mM EDTA in 10 mM Tris•HCl, pH 8.0). This sample was counted to provide a measure of *TdR retained within intact DNA.

Radioactivity was counted in a Packard Tri-Carb scintillation counter (Packard Instrumentations Co., Downers Grove, IL). Percent *TdR released was determined using the formula:

$$\%\ ^*\text{TdR released} = \frac{(\text{cpm in [(cpm in medium) + fragmented DNA)]})}{\text{total cpms in all fractions combined}} \times 100$$

Morphology and Propidium Iodide Staining:

Cultures were examined using phase-contrast microscopy for general morphology. In addition, in some cultures incubation medium was replaced after 2 days with PBS containing 5 µg/µL propidium iodide (PI, from Molecular Probes, Inc., Eugene, OR). Following a 10-minute incubation at 4°C in a light-proof box, PI was removed and slide chambers were then washed three times before mounting in a medium consisting of 1% p-phenylenediamine in PBS and glycerol (1:9, v:v) adjusted to pH 8.0. Specimens then were viewed using a Jenalumar fluorescence microscope (North Central Instruments, Inc., Plymouth, MN).

DNA Agarose Gel Electrophoresis:

Following exposure of myoblasts to early or late WFs or to differentiation medium for 2, 24, or 48 hours, DNA fragmentation was evaluated using the method of Wright et al. (1993). Briefly, fragmented DNA was extracted initially and collected in the same manner as was used of measuring *TdR release, above. However, DNA in the supernatant was further purified and subjected to gel electrophoresis as follows: DNase-free ribonuclease (1 ng/mL) was added to the supernatant which was then incubated for 1 hour at 37°C. DNA subsequently was collected by phenol:chloroform (1:1, v:v) extraction and ethanol precipitation. The resulting DNA pellet was re-solubilized in suspension buffer (as above) and electrophoresed in a 1% agarose gel containing 0.5 mg/mL ethidium bromide.

Cultures of bovine pulmonary endothelial cells (CCL-209; ATCC) exposed for 6 hours to tumor necrosis factor-alpha (20 ng/mL) were used as positive controls for DNA fragmentation. These cultures were kindly provided by Dr. Peter Bitterman (Department of Medicine, University of Minnesota).

Reagents, Radioisotopes, and Media

All chemical and biochemical reagents used in these studies were purchased from Sigma Chemical Co., (St. Louis, MO) unless otherwise specified. Tissue culture media, PBS, and serum were purchased from Gibco BRL (Grand Island, NY).

Statistical Analyses

Three to five replicates of assays were performed to generate each data point. Data are expressed as means ± 1 sem. Statistical analyses consisted of one-way analysis of variance (ANOVA) and unpaired t-test. Initial analyses of composite treatment effects were performed by ANOVA. Subsequent comparisons between groups were performed using t-tests. Differences were considered significant at $p < 0.05$; no further refinement of significance was made. Computations were performed using StatView™ (BrainPower, Inc., Calabasas, CA).

Results and Discussion

Regeneration requires expansion early after injury of either a stem cell or a parenchymal cell population and the subsequent reintegration of these cells as functional elements of the adult tissue. Sicard and Nguyen (1994) suggested that the repair environment associated with PVA sponges is not supportive of skeletal muscle regeneration. This was based on the failure of WFs to support fully the above stated expectations. Although early WFs were adequately mitogenic for myoblasts *in vitro*, late WFs did not promote myoblast differentiation *in vitro*. The failure of late WFs to specifically promote either proliferation or differentiation of myoblasts raised the question of whether late WFs might have yet other effects on myoblasts.

Overnight incubations in WFs affected recovery of L8 myoblasts (Table 1). Compared to the number of cells recovered following incubation in base medium (DMEM plus Medium 199, 4:1 [v:v]) to which neither serum nor WFs was added, exposure to WFs harvested

TABLE 1: MYOBLAST RECOVERY (PERCENT OF CELLS PLATED) FOLLOWING 24-HOUR INCUBATION

Calf Serum	Wound Fluids				
	1-day	3-day	5-day	10-day	15-day
100 ± 1	122 ± 10*	114 ± 6*	100 ± 10	73 ± 4*	56 ± 5*

Serum or WF was added to base medium (DMEM + Medium 199; 4:1, v:v) to achieve a final concentration of 10% (v:v). Results are mean ± 1 sem (n = 3 – 5). * $p < 0.05$

1 or 3 days postimplanted increased expected cell recovery approximately 14% and 22%, respectively ($p < 0.05$). In contrast, WFs harvested 10 or 15 days postimplantation decreased expected recovery by approximately 27% and 44%, respectively ($p < 0.05$). During this short incubation period, 5-day WF had no effect on expected cell recovery.

All cells were plated under identical conditions and routinely displayed plating efficiencies greater than 90%. Cell counts were performed in a manner that would enable all cells present within each well to be counted, whether attached or floating. Differences in cell numbers under these conditions suggest that 1- and 3-day WFs supported, or promoted, proliferation but that 10- and 15-days WFs might have induced cell death.

In order to further explore this question, we measured release of *TdR from prelabeled cells. Cells exposed to late WFs released substantially more *TdR that did cells exposed to early WFs or maintained in growth or differentiation media (Table 2). Very little *TdR was released (1.8 ± 1.1%) by cells immediately after transfer to test conditions. Maintaining myoblasts under growth conditions (i.e., medium containing 10% serum) yielded 3.8 – 4.6-fold increase in *TdR release over the ensuing 48 hours. Under differentiation conditions (i.e., in medium without serum or growth factors), mean release of *TdR was slightly greater, up to 7.3-fold, over this same interval. Exposure to early WF (1-day postimplantation) yielded a very similar effect; 11.9 ± 3.4% and 8.8 ± 1.0% *TdR released at 24 and 48 hours, respectively. On the other hand, exposure to late WFs (10- or 15-days postimplantation) markedly increased *TdR release. In particular, *TdR release was 18.7 – 20.2-fold greater than that seen at t_0 and was 2.8 to 4.8-fold greater than that observed for the other conditions after 24 or 48 hours' incubation.

Wang and Walsh (1996) reported that approximately 16% of myoblasts in cultures of murine C2C12 cells were undergoing apoptosis within 24 hours of serum deprivation and that between 20 and 30% of these cells were undergoing apoptosis after 48 hours. These observations are in good agreement with the present observations (Table 2). In addition, the greater incidence of cell death displayed by murine C2C12 myoblasts suggests that this myoblast cell line might be more sensitive than L8 cells to serum withrawal or growth factor deprivation. Since our assay is biased towards evaluating changes in cell proliferation rather than total cell population, differences in promotion of cell death following serum withdrawal between these two cell lines might be even greater.

While it seems that slightly more than 10% of proliferating L8 cells showed signs of death following

TABLE 2: WOUND FLUID EFFECTS ON [^{3}H]-THYMIDINE RELEASE FROM PRELABELED MYOBLASTS

Condition	[^{3}H]-thymidine release (% of total)		
	0 hours	24 hours	48 hours
growth medium (contains 10% calf serum)	1.8 ± 1.1	7.0 ± 1.6	8.3 ± 4.4
differentiation medium (no serum or WF)		11.1	13.1
1-day WF		11.9 ± 3.4	8.8 ± 1.0
10- or 15-day WF		33.6 ± 4.2*	36.3 ± 2.9*

Serum or WF was added to base medium (DMEM + Medium 199; 4:1, v:v) to achieve a final concentration of 10% (v:v). Results are mean ± 1 sem (n = 3 – 5). * $p < 0.05$

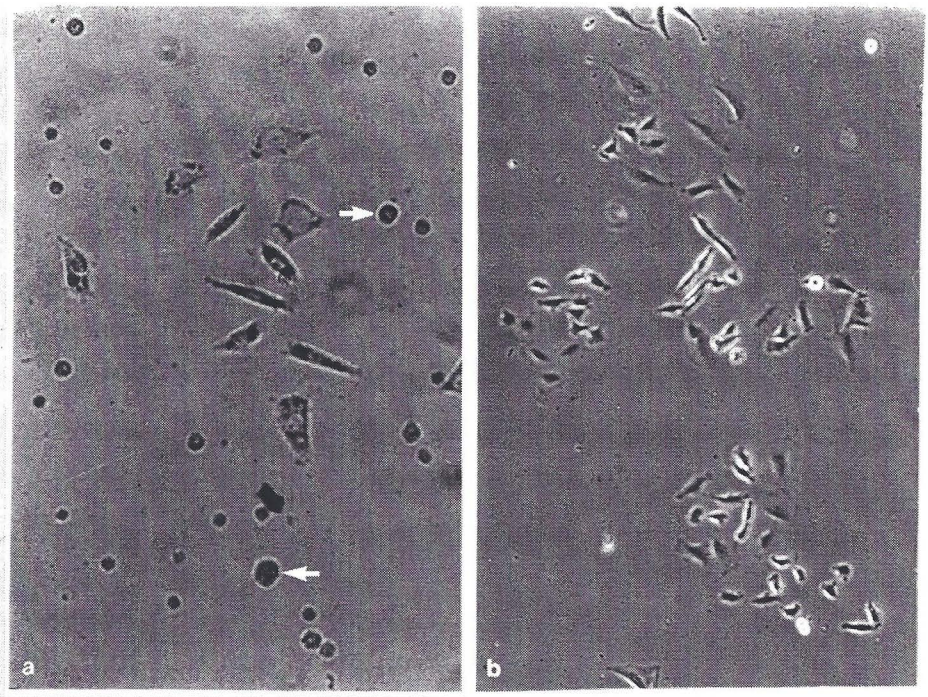

Figure 1: Rat neonatal L8 myoblasts become rounded and detach (indicated by arrows) when exposed to 10- or 15-day wound fluids (a) but remain attached to the substrate in the presence of 1-day wound fluid (b).

serum deprivation, nearly three times as much cell death occurred following exposure to late WFs (Table 2). This suggests that late WFs not only might prevent myoblast proliferation, as demonstrated previously (Sicard and Nguyen, 1994), but also might actively promote cell death. Under these circumstances, cell death could be the result either of apoptosis or necrosis. *In vivo*, necrosis affects pockets of neighboring cells and stimulates inflammation, whereas apoptosis affects individual cells and typically does not provoke inflammatory events (Schwartzmann and Cidlowski, 1993). Discriminating between these two modes of cell elimination within this environment, *in vivo*, will be important for understanding the physiological dynamics occurring in the late wound environment and to characterizing potential differences between repair and regeneration microenvironments.

When examined microscopically, characteristics consistent with apoptosis were apparent following exposure to late, but not early, WFs. For example, within 24 hours exposure to 15-day WFs, cells became rounded (Figure 1a) and often detached. In addition, many displayed obvious cytoplasmic blebbing when viewed by phase-contrast microscopy (data not shown). Cells under growth (serum or 1-days WF) or differentiation (serum-free medium) conditions did not display this phenomenon (Figure 1b). Furthermore, the frequency of nuclei stained with PI in cultures of nonpermeabilized cells was perceptibly greater following exposure to 15-day WF (Figure 2a) as compared to exposure to 1-day WF or serum (Figure 2b). Figure 2a depicts 800 – 850 cells (allowing for the inability to discriminate discrete nuclei in certain portions of the field) of which 39 fluoresce brighly. This indicates that 4.6 – 4.9% of the cells have nuclei stained with PI. In contrast, Figure 2b depicts approximately 600 – 625 cells of which only 4 fluoresce strongly; i.e., only 0.6 – 0.7% of cells are PI-positive. This represents a 6.6- to 8.2-fold greater degree of fluorescent nuclei in the presence of late WFs than early WFs. These observations indicate that cell integrity has been compromised by exposure to late WFs.

Evaluation of cellular extracts on agarose gels failed to provide evidence of endonuclease-mediated DNA fragmentation (data not shown). While the presence of a DNA fragmentation ladder is a common finding with cells undergoing apoptosis (Schwartzmann and Cidlowski, 1993), it is not a universal event. For example, cell death in the interdigital regions of embryonic mouse limbs and in the labial glands of tobacco hornworm larvae is not accompanied by demonstrable DNA fragmentation ladders (Zakeri et al, 1993).

The results of the present study add another property to that already demonstrated for late WFs. Thus, the interstitial fluid for the late wound environment (i) is not mitogenic for fibroblasts (Pricolo et al, 1990) and myoblasts (Sicard and Nguyen, 1994),

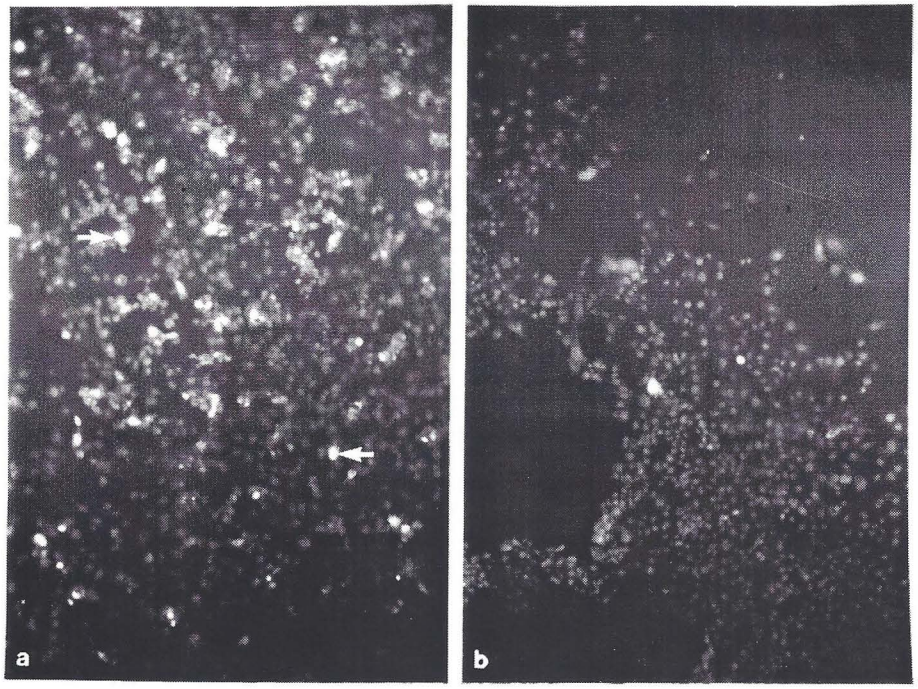

Figure 2: Nuclear labeling of nonpermeabilized L8 myoblasts with propidium iodide (indicated by arrows) following exposure to 15-day (a) or 1-day (b) wound fluid.

(ii) does not promote myoblast differentiation (Sicard and Nguyen, 1994), and (iii) promotes or induces myoblast death, *in vitro*.

At present it is unclear what mechanism(s) might promote myoblast death in culture following exposure to late WFs. Defects in or alterations to intercellular signaling (Raff, 1992; Evan and Littlewood, 1998) and in cell-matrix interactions (Frisch and Francis, 1994) are believed to contribute to activating programmed cell death. According to these hypotheses, continued cell survival depends upon autocrine or paracrine signals from neighboring cells or anchorage with the substrate that balances proliferation and differentiation against apoptosis. Depriving cells of their normal input activates mechanisms leading to death unless alternative protective measures are invoked. Frisch and Francis (1994) suggest that protection is conferred upon epithelial cells following anchorage disruption produced by injury through substitution of new interactions with the modified substrate environment of the wound. Moreover, the state of differentiation of a cell might affect whether death ensues following exposure to conditions or environments that are potentially death-inducing. Illustrating this point, Wang and Walsh (1996) disclosed that myotubes and differentiating myocytes, but not myoblasts, of the murine C2C12 cell line were resistant to apoptosis induced by serum deprivation. Resistance to apoptosis correlated with the expression of cyclin dependent kinase inhibitor $p21^{CIP1}$, associated with cell cycle withdrawal, but not with myogenin, a basic helix-loop-helix protein expressed earlier in myoblast differentiation.

The differential sensitivity of myoblasts and myocytes to apoptosis-inducing conditions (Wang and Walsh, 1996) enables us to potentially reconcile the results of the present study conducted *in vitro* with a previous study conducted *in vivo* (Sicard, Nguyen, and Witzke, 1997). In that study, viable transfected L8 cells were found in PVA sponges 15 days after implantation. Although these cells remained mononuclear (i.e., they did not differentiate morphologically into myotubes), they expressed immunoreactive α-sarcomeric actin. Presence of this muscle-specific gene product suggests that these cells might have been proliferating myocytes rather than myoblasts.

Inducing programmed cell death, or apoptosis, in the late wound environment might represent an important aspect of achieving resolution to injury (Desmoulière et al., 1995; Savill, 1997). Through programmed cell death, macrophages and other inflammatory cells as well as excess cycling fibroblasts might be removed from the site of active repair. Similarly, programmed cell death might assure removal of activated stem cells or parenchymal precursors that have not differentiated and/or become effectively reintegrated into a tissue or organ undergoing regeneration.

We have recently noted that the inflammatory responses associated with our regeneration model are more moderate than those in our repair model (Sicard, 2000; Sicard and Mand, 2000a, 2000b). This raises questions about the cytokine composition of fibrotic wound repair and tissue regeneration microenvironments since cytokines are important modulators of inflammatory and immunological responses (Horuk, 1998; Gerard and Rollins, 2001; Moser and Loetscher, 2001) and the property of WFs inducing apoptosis is apparently associated with one or more soluble factors. Products of these inflammatory cells, as they paticipate in either repair or regeneration, also are candidates for inducing death of the myoblasts in this study. Further investigation clearly is warranted to define the component(s) of late WFs responsible for promoting myoblast cell death in vitro, to characterize the mechanism(s) through which these factors act, and to more fully establish the relevance of this phenomenon to postinjury resolution in vivo.

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