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Cellular anoxia constitutes an environmental stimulus experienced within early stage healing wounds. Anoxia has effects on fibroblasts which make important contributions to regulating the progressive events of wound healing. These effects are mediated through specific gene induction, resulting in activation of a cellular program including enhanced glycolysis, protease secretion, endonuclease production, and expression of an angiogenesis factor. In a delayed response, fibroblast contractile processes activate at a time appropriate for wound contraction.

The anoxic fibroblast response is constitutively activated in a large fraction of malignancies, where it contributes to invasiveness and genomic instability.

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V. A. Introduction.

1. Wound healing.

Wound healing represents an intricately regulated symphony of several cell types playing their individual roles in a carefully coordinated manner with regards to both time and space. Much of the coordination is effected through the interplay of secretory cytokines, although other elements including cell-cell contacts and interactions with the extracellular matrix also play significant roles (1,2). The studies described in this proposal are designed to elucidate the role of an unusual regulatory signal in wound healing, oxygen tension, and specifically focus on the effects of anoxia and reoxygenation on the activity of fibroblasts within the healing wound.

Wound healing occurs in a series of stages. Following the initial trauma, clotting occurs with fibrin forming the first matrix within the wound. Neutrophil infiltration begins within approximately ten minutes and occurs for about one day, and is followed by macrophage infiltration. Fibroblast infiltration begins on the first day and continues for roughly three or four days. These early events are combined in an inflammatory phase associated with wound debridement and resistance to infection. After around five days fibroblasts are actively replicating and secreting collagen, and neovascularization is occurring. Around seven to ten days, wound contraction occurs through the action of contractile fibroblasts known as myofibroblasts. Wound remodeling occurs over the next month as collagen fibers are restructured and wound strength is increased.

Several regulatory elements active in the early stages of wound healing have been identified. Platelets release platelet derived growth factor which is chemotactic for subsequent cells and also facilitates their growth. Macrophages secrete a multitude of growth factors and lymphokines, including PDGF, FGF, TGFα, TGFβ and IL-1;

IL-1 appears to induce fibroblasts to produce additional PDGF (3,4,5). In addition, macrophages secrete an angiogenesis factor (TNFα) whose secretion is regulated by the oxygen tension within the wound, and occurs only under anoxia (6,7).

Fibroblasts have several important roles in wound healing. Fibroblasts migrate into the wound but do not themselves divide until neovascularization occurs. In the early stages of wound healing, fibroblasts secrete matrix metalloproteinases including collagenases, stromelysin, gelatinase, and plasminogen activator (8). In an anoxia induced response, we have shown they additionally secrete the proteases cathepsins D and L (9). We have shown endonucleases are also induced by anoxia and appear to be secreted (10). Some fibroblasts differentiate into myofibroblasts rich in internal actomyosin filaments, whose action underlies much of the phenomenon of wound contraction (11).

What regulates fibroblast activity within the wound? Fibroblasts respond to several growth factors secreted by platelets and macrophages. Wound lactate affects fibroblast collagen production (12). Studies we have carried out further establish that fibroblasts are regulated to a major degree by oxygen tensions, and we have shown this occurs in wound healing (13,14). Less is known of what signals fibroblasts use to communicate to each other and to other cell types within the wound and in the periphery, although such signaling should be particularly important in regulating subsequent events in wound healing. Two known signaling factors secreted by fibroblasts in the wound are PDGF and TGFβ, which helps effect wound contraction (15,16).

Oxygen tensions vary dramatically within the wound environment (17,18). Within the center of the wound, essentially anoxic conditions prevail; this unvascularized region is characterized by high lactate and CO_2 concentrations and low pH. Macrophages and fibroblasts

surround this zone; the metabolic activity of such cells consumes what little oxygen is present. The anoxic conditions stimulate angiogenesis factor production by macrophages, promoting neovascularization with a subsequent resultant increase in oxygen tension. Prevention of anoxia slows neovascularization and impairs subsequent healing (19). At the edges of a wound and in the surrounding tissues, aerobic conditions prevail. Even in surface wounds such as burns essentially anoxic conditions exist, since relatively little surface atmospheric oxygen can diffuse into the wound. In the absence of a clear understanding of the regulatory role oxygen plays at the molecular and cellular level, attempts at influencing wound healing by altering exogenous oxygen levels appear to be somewhat premature (20).

B. The response of fibroblasts to anoxia.

Fibroblasts are a major component of the wound healing process, and fibroblasts function under anoxic conditions in the early stages of wound healing. Studies predominantly in our laboratory have demonstrated how anoxia plays a major regulatory role for fibroblasts. Our work has revealed that the response of fibroblasts to anoxia occurs in stages (Table I). The first observed response, occurring within around two to three hours, is induction of retrotransposon-related SVL30 RNA (13,14,21,22). This initial induction of five- to ten-fold is protein synthesis independent; a secondary wave of induction occurring after six to eight hours is dependent on new protein synthesis and typically reaches 50-200 fold (figure 1). Cell DNA replication ceases after six hours, but RNA and protein synthesis continue, and the cells remain viable for 3 or 4 days of anoxia. Metabolism shifts to glycolysis. Seven major intracellular proteins are induced after about six to eight hours; these include the glucose regulated proteins grp94 and grp78, and the anoxia inducible lactate dehydrogenase LDH-k (23). Two known transcription factors appear to be

transcriptionally induced: C/EBPβ and ATF-4 (figure 2). After around twelve hours, four major secretory proteins are induced: p61, p51, p40, and p22 (9,13). The secretory p40 has been identified as the protease cathepsin L, a protease which is also known to be induced following injury (24,25), and the p51 is the protease procathepsin D. Both cathepsins are most active in an acidic environment such as provided by cells actively secreting lactate. Fibroblast viability continues for three to four days, as measured by colony forming efficiency and trypan blue exlusion (10).

During wound healing, some fibroblasts mature into myofibroblasts rich in actomyosin filaments (11). After roughly seven to ten days myofibroblasts contract, drawing the edges of the wound closer together (26,27). Such contraction occurs following neovascularization, under aerobic conditions. In what appears to parallel this <u>in vivo</u> response, we have observed that fibroblasts which have been anoxic more than 18 hours and then returned to aerobic culture undergo a contractile response about one week later (figure 3). There is again no associated loss of cell viability. This delayed contractile response is apparently mediated by a secretory factor or factors produced only during the first two days following reoxygenation; culture fluid from such fibroblasts causes other fibroblasts which have never been anoxic to undergo contraction after a similar one-week latent period. The delayed nature of the contractile response differentiates the anoxia induced secreted factor from TGFβ and endothelin which elicit contraction within a few hours (15,16,28).

The studies we propose are aimed at understanding the nature of oxygen regulation of fibroblast activity, and the role this plays in wound healing.

Table I. The Staged Response to Continuous Anoxia by FRE Rat Fibroblasts (updated from Anderson et al. (13,14)

Time into Anoxia	Phenomenon
2-3 hr	SVL30 RNA induction (5-10X)
6-8 hr	Cessation of DNA synthesis and PCNA synthesis
	Induction of 2 transcription factors: C/EBPβ and ATF-4
	Induction of 7 major intracellular proteins related to glycolysis and glucose utilization, including p34 (LDH_K), glucose regulated proteins (grp94 and grp78), p61 (potentially SVL30 encoded), p56, p47 and p21
	Sharp rise in SVL30 RNA transcription (ca 100X)
12 hr	Production of four major secretory proteins p22
	p40: cathepsin L
	p51: cathepsin D
	p61: (protease)
16 hr	Intracellular endonuclease appears
72 hr	Fibroblast viability begins to decline; all cells dead by 120 hr
Fibroblast Responses to Reoxygenation Following 48 Hours of Anoxia	
0-1 hr	translation temporarily blocked
1 day	cell cycling resumes
1-2 days	secretion of contraction inducing activity
7-10 days	cell contraction occurs; rope-like forms

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VI. Body: The Anoxic Fibroblast Response in Wound Healing.

Fibroblasts respond to temporary anoxia in a complex fashion which includes induction of seven major intracellular proteins and at least three major secretory proteins [1]. As defined in the FRE rat fibroblast model system, the response occurs in a stepwise fashion during the first two days of anoxia and is associated with no loss of cellular viability [2]. Biochemical features suggested the anoxic response might be appropriate for a physiological role of fibroblasts during the debridement stage of wound healing, although no direct experimental evidence has existed for such a role. Since the anoxic fibroblast response results in induction of retrotransposon SVL30, secretion of cathepsin L, activation of a sequestered (and presumed secreted) endonuclease, and a metabolic switch to glycolysis, and these same features are also frequently coordinately seen in malignant tumor cells, it has further been proposed that appropriate or inappropriate expression of this response may significantly contribute to malignancy [3].

Although fibroblasts infiltrate wounds soon after injury, their principal known roles in wound healing have been in middle and late stage events, in collagen secretion and wound contraction [4-6]. With essentially anoxic conditions known to prevail within the interior of early stage healing wounds, the potential has existed for active involvement of the anoxic fibroblast response in wound healing [8-11]. However the effects of low oxygen tensions on wound fibroblasts have not previously been examined, nor has expression of

the anoxic fibroblast response during wound healing.

To directly assess if the anoxic fibroblast response is physiologically expressed during wound healing, we have used the polyvinyl alcohol sponge model of Davidson to monitor in vivo induction of SVL30 RNA and several anoxic response proteins [12-14]. We have found that SVL30 RNA is strongly induced during the early anoxic phase of wound healing. A newly identified anoxic response protein, procathepsin D, was also found highly expressed within healing wounds. A particularly surprising effect of anoxia on fibroblasts was a delayed cellular organizational response which has the potential to participate in wound contraction.

1. Methods

Cell Culture

The FRE rat fibroblast line was originally obtained from the Laboratory of Stuart Aaronson, NIH. Cells were cultured in DMEM (1000 mg glucose/l) containing 10% calf serum. Anaerobic culture was performed in a Forma anaerobic glove box incubator (Marietta, OH) with an additional blower/palladium catalyst system added to expedite trace oxygen removal; the atmosphere was 85% ultrapure nitrogen, 10% hydrogen, and 5% carbon dioxide.

Metabolic Labeling of Proteins

Cells were plated at 2 x 10^6 cells per 10 cm dish one day before being placed in the anaerobic chamber. At the time of labeling, cells were rinsed twice with anaerobically equilibrated

methionine free media containing 2% dialyzed calf serum (Gibco, Grand Island, NY). Labeling was in 3 ml/dish of this same media supplemented with 0.1 mCi/ml ^{35}S methionine plus cysteine (NEN Express, Boston, MA), for a period of 4 hours for intracellular protein analyses and 40 minutes for secretory proteins. Intracellular protein extracts were harvested immediately as described (1), with the exception that 400 IU/ml trasylol were added at the time of cell lysis. Secretory proteins were collected by removing the ^{35}S containing media, rinsing twice with 5 ml DMEM per dish, and then adding 3 ml DMEM per dish. After 3.5 hr this media was harvested and processed as described [1].

Immunoprecipitation Analyses

Antisera to cathepsin D were obtained from John Chirgwin and from Bio Rad Laboratories, Hercules, CA. Immunoprecipitations were carried out by reacting approximately 10^7 cpm of intracellular proteins or 10^5 cpm of secretory proteins with 2 µl antisera in 250 µl of buffer containing 2% triton X-100, 0.5% SDS, 0.05M Na_2HPO_4 pH 7.4, and 100 µg/ml ultrapure bovine serum albumin (Sigma, St. Louis, MO). After 18 hours incubation at 4^o, 15 µl of protein-A sepharose beads (Pharmacia, Piscataway, NJ) was added, followed by gentle agitation for 30 minutes. Beads carrying the bound immune complexes were centrifuged momentarily at 10,000 g, the supernatant removed, and washing repeated four times in the immune precipitation buffer without BSA. A fifth wash was in 0.01M Na_2HPO_4 pH 7.4, and then the beads were suspended in SDS gel sample buffer and analyzed on SDS-

PAGE as described [1].

Experimental Rat Wounds

Twelve week old female F-344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Experimental wounds consisted of 3 x 3 x 20 mm polyvinyl alcohol sponges surgically implanted beneath the ventral panniculus carnosus muscle; the wounds were closed with chromic sutures. Sponge material (Unipoint, High Point, NC) was washed extensively and autoclaved in water before use. At the indicated time points animals were euthanized with ether, the sponge and spleen removed, and both were stored under liquid nitrogen prior to RNA and protein extractions. These studies were carried out as per RPCI protocol 038R, approved by the Roswell Park animal care and use committee.

RNA Extraction and Analysis

RNA was isolated with guanidinium thiocyanate by the method of Puissant and Houdebine [15]. Slot blot analyses were as previously described [12]. Blots were probed for SVL30 expression using the 1.3 kb subclone pVL30DS [1].

1. Results

The Anoxic Fibroblast Response is Expressed During Wound Healing

Although we have suggested that the anoxic fibroblast response might be a wound healing response, direct evidence has been lacking [1, 3, 12]. Since the initiating SVL30 gene is strongly induced at

the transcriptional level in anoxic fibroblasts, analyses at the RNA level can be used to directly examine if this feature of the anoxic fibroblast response is activated within healing wounds [12, 16]. In order to obtain sufficient wound tissue RNA for analysis and to ensure that a large enough wound volume would exist to generate anoxic conditions, we utilized the wound model system of submuscular implantation of polyvinyl alcohol sponges [13]. 3x3x20 mm sterile sponges in water were implanted beneath the ventral panniculus carnosus of twelve week old female Fischer F-344 rats. The wounds were allowed to heal for periods of 1 to 180 days, after which the animals were sacrificed and the sponges containing granulation tissue were removed and quickly frozen in liquid nitrogen for subsequent RNA analyses. Spleens were also collected as a representative control tissue [17]. In parallel, other rats were anesthetized, the wound opened, and oxygen tension within the sponge immediately measured at several points by inserting an oxygen microelectrode contained within a 22 gauge needle (Diamond General). As shown in figure 1 and table 1, SVL30 RNA was induced by around 10-fold within the wound on days 1 and 2. Since SVL30 RNA has a six hour intracellular half life under both aerobic and anoxic conditions [16], this expression must represent continued elevated rates of synthesis. In contrast to wound tissues, SVL30 RNA levels varied less than 2-fold in the spleens of these animals. Oxygen tension measurements showed the pO_2 within the wound was less than 15 mm Hg on day 1, while reaching levels over 50 mm Hg for all time points on or after day 4.

We also examined expression within the wound tissues of the p34

anoxic response protein which is expressed de novo in anoxic fibroblasts [18]. This protein was measured by assaying its associated k-isozyme lactate dehydrogenase activity. As shown in figure 2A, this protein was expressed in cultured rat fibroblasts by sixteen hours into anoxia, and was abundant in wound tissue on day 2 (figure 2B). An analogous situation appears to exist in human wound healing, where LDH-k can be seen in circulating serum about one week postoperative (figure 2C).

Multiple Proteases Including Procathepsin D Are Secreted By Anoxic Fibroblasts

Zymography provides a convenient means to separate multiple enzymes with similar activities, and simultaneously provides a quantitative indication of the activity of each enzyme. We used protease zymography to evaluate if proteases in addition to the previously identified procathepsin L [1] might be secreted by anoxic FRE rat fibroblasts. Figure 3 shows two protease activities are secreted in response to twenty or more hours of anoxia. These bands migrate with sizes of 42 and 47 k. The lower band is consistent with the size and anoxia responsiveness of procathepsin L [1]. Previous studies with ^{35}S methionine labeling showed three major bands of anoxia inducible secretory proteins, with two of similar size to the anoxia inducible proteases, and such labeled secretory proteins appear at the same time as these proteases [1]).

With the p40 anoxia inducible secretory protein already identified by us as the protease procathepsin L, we sought to

determine if the major secretory protease of 47 kd might also represent a cathepsin. Procathepsin D was a candidate, since it has two proenzyme forms with molecular weights of ca 46 and 44 kd [19], and a secreted form reported about 52 kd (20). ^{35}S methionine labeled secretory proteins were compared to intracellular proteins reacted with antisera to cathepsin D, and an anoxia inducible ^{35}S secretory 51 kd protein migrated at the same position as immunoprecipitated procathepsin D (figure 4A). This position was very similar to the major protease band shown on zymography. The anoxia inducible secretory p51 was further found to be immunoprecipitated by antisera to cathepsin D (figure 4B). Peptide analyses were carried out using Cleveland gels [21] to compare the V-8 protease cleavage peptides of the secretory p51 with those of cathepsin D (figure 4C). The V-8 digest of the secretory p51 was found to have four out of five cleavage peptides in common with the 51 kd form of cathepsin D, and thus appears to represent a form of cathepsin D. The smallest cleavage peptide of cathepsin D appeared to undergo one additional cleavage in the secretory form of p51, which may reflect an additional posttranslational modification or altered transcriptional start site relating to the secretion process itself.

What causes cathepsins D and L to be secreted at elevated levels by anoxic fibroblasts? One possibility was that transcription of these genes is elevated. This was examined by slot blot analyses, which revealed that neither cathepsin D (figure 5A) nor cathepsin L (data not shown) showed elevation of their mRNA levels. Examination

of ^{35}S methionine labeled extracts reacted with antisera to cathepsin D (figure 4A) and cathepsin L (data not shown) also revealed no major change induced by anoxia in the intracellular levels of these proteins. Total production of these proteases is thus evidently not altered significantly within anoxic fibroblasts, but instead a fraction of these normally lysosomal enzymes becomes rerouted outside the cell. Possible factors involved in such rerouting are 78 and 74 kd proteins seen in immunoprecipitates of cathepsin D from anoxic fibroblasts, but not seen with extracts of aerobic cells (figure 5B). Since these proteins are not immunoprecipitated in extracts which have been subjected to prior heating, they are evidently complexed with cathepsin D but not directly recognized by the antibody itself. Although the grp78 which appears in anoxic fibroblasts would be an attractive candidate to effect such rerouting, we have been unable to show immunoreactivity with such antisera.

Anoxia Primes a Delayed Cellular Contractile Response

In a wound environment, fibroblasts experience anoxic conditions for only a few days, after which neovascularization restores aerobic conditions [9, 10, 11]. Fibroblasts within the wound then secrete collagen which is an essential matrix component for subsequent wound contraction and scar formation [7, 22]. Analysis of secretory proteins produced by fibroblasts returned to an aerobic environment reveals a major shift to high level procollagen p140 secretion occurs within two days (figure 6). At this point secretion of the anoxia inducible secretory proteins has

ceased.

Approximately one week after wounding, wound contraction occurs in a response involving contraction of myofibroblasts [7, 23, 35]. It has never been clearly established if such cells derive from a new class of fibroblasts which infiltrate the wound relatively shortly prior to contraction, if they represent a newly differentiated state of fibroblasts which have infiltrated the wound much earlier, or if they represent some combination of the two. We have observed that fibroblasts which experience anoxia for as little as 16 hours are primed to undergo a major cellular contractile response approximately 7-10 days later (figure 7). This results in the formation of rope-like lattices, which appear to represent differential axes of cellular adhesion and contraction. Culture media from fibroblasts recovering from anoxia elicits the same response in other fibroblast cultures which have never experienced anoxia (figure 8). These results suggest either that (i) a factor is secreted by recovering fibroblasts, which mediates the contractile response in fibroblasts which have experienced anoxia and probably also in fibroblasts which have infiltrated after the anoxic phase, or (ii) that fibroblasts recovering from anoxia selectively remove some component which normally serves to block contraction. Mixing studies with conditioned and fresh media indicate the contractile response is dominant, supporting the first possibility.

VII. Conclusions

Previous studies of the anoxic fibroblast response have focused

on its biochemistry and gene regulatory patterns; the present study provides a physiological context for the response. Wound healing is closely regulated through an interconnected network of signaling peptides [24], but the massively shifting oxygen levels present during wound healing are also evidently utilized in regulating cellular activities [11]. Previous studies have shown anoxia induces macrophages to express TNFα, which has an angiogenic activity [25, 26], and epithelial cells induce EGF receptor in response to hypoxia [27]. The inducibility of vascular endothelial growth factor by anoxia in several cell types points to a role in wound healing [38]. Fibroblasts can now be added to the list of anoxia responsive cells in the wound, specifically inducing protease and nuclease (2) secretion appropriate for wound debridement.

The contractile response induced in fibroblasts a week after experiencing anoxia would appear appropriate for wound healing at the stage of wound contraction. It is interesting that fibroblasts experiencing 8 hours or less of anoxia exhibit no or very little such contraction, while 16 hours or more suffices to induce the delayed response 7 or more days later. The molecular basis of this observation needs to be elucidated, including evaluation if a new differentiation state is induced in the fibroblasts. The delayed nature of this contractile response distinguishes it from the rapid responses elicited by such factors as endothelin or TGF-β, which elicit contraction within twelve hours [28], although such factors could still be involved in anoxically primed cells at that time when actual contraction is occurring. Low oxygen tensions playing a major

regulatory role in the wound contraction response would be consistent with the observation by Hunt that wounds maintained under highly aerobic conditions through use of gas permeable membranes fail to contract properly [29].

Key questions remain as to how the anoxic fibroblast response is initiated, regulated and staged. For example, while SVL30 is transcriptionally induced very early, cathepsin secretion is late and does not reflect simple transcriptional induction of the cathepsins themselves. Cathepsin secretion evidently results from appearance of a rerouting activity in the later stages of the response; the possible role of the co-precipitating 78 and 74 kd proteins in the rerouting process is currently being investigated. Definition of the molecular basis of the anoxia responsiveness of the fibroblast program especially requires detailed characterization of those genes which directly respond to low oxygen tensions. The SVL30 element which responds rapidly to anoxia appears to in turn induce many other secondary anoxia responsive genes; recent work in our laboratory has defined an anoxic response DNA sequence residing within the SVL30 LTR sequences (3, S.D. Estes, unpublished observation). Limited evidence suggests that a transactivation mechanism similar to that utilized by lentiviruses such as HIV and HTLV-1 might be used by SVL30 to induce some of the secondary anoxic response genes, although the induction of several bZIP transcription factors during the secondary response indicates overall regulation and staging is quite complex [3, 30, 31]. Based on the limited coding capacity of known SVL30 elements, SVL30 appears incapable of

encoding more than two or three proteins in the 10 kd range [37]; most components of the anoxic fibroblast response are other cellular genes which become secondarily activated.

Our results would suggest that interfering with the establishment of anoxic conditions in the inflammatory phase of wound healing should disrupt debridement occurring simultaneously and contraction occurring subsequently. While this would be most undesirable for normal wound healing, it may offer useful approaches for dealing with wounds healing improperly, particularly with excessive protease action [35]. Pressure ulcers might be expected to be especially susceptible to such intervention, particularly since tissue hypoxia appears to contribute to their formation [36]. Similarly, ensuring or augmenting a transiently anoxic environment may prove useful in ensuring that subsequent contraction occurs.

Malignancy shares key features with the anoxic fibroblast response, and loss of overall control of the response has been postulated to be a significant event in tumor progression [1, 3]. The wound healing system may now yield novel insights and therapeutic approaches to malignancy by revealing how the anoxic response can be terminated, as it is during wound healing prior to neovascularization and restoration of aerobic conditions. This would especially be the case for tumor cells which have lost the oxygen sensing induction mechanism utilized during wound healing, while retaining the essential components of the shut-off system.

Table 1

SVL30 and Cathepsin D Expression
in Experimental Wounds

Day	Number of rats	SVL30 RNA (a) Wound	Spleen	Fold Induction	Wound Cathepsin D (b)
1	4	7.02	0.51	13.7	0.16 ± 0.15
2	5	3.22	0.38	8.5	1.00 ± 0.19
3	4	1.20	1.11	1.1	0.72 ± 0.03
4	6	2.76	1.38	2.0	0.25 ± 0.19
5	3	0.52	0.49	1.1	0.03 ± 0.03
7	4	0.86	0.46	1.9	0.00
11	2	1.39	1.15	1.2	0.00
180	2	0.28	1.05	0.3	0.00

(a) SVL30 RNA was quantitated by slot blot analyses, as in figure 1, and compared to spleen values from the same animal. Data is expressed in integrating densitometer units, with the average of all 30 spleens being 0.81 densitometer units.

(b) Cathepsin D was assayed by western blot assays. Values are normalized to day 2 wound tissue. Spleen showed no measurable cathepsin D. Western blot assays were by the method of Harlow [34].

FIG. 1. SVL30 RNA is strongly expressed within healing wounds during the early phase of wound healing. RNA was isolated from 3x3x20 mm polyvinyl alcohol sponges implanted beneath the ventral panniculus carnosus of twelve week old female F344 rats, and slot blots were analyzed for SVL30 expression with the probe pVL30DS. Spleen RNA prepared from each animal was used as a control. SVL30 expression was quantitated by densitometric scanning and is expressed relative to the average of all spleens. Data is from a representative experiment of four total.

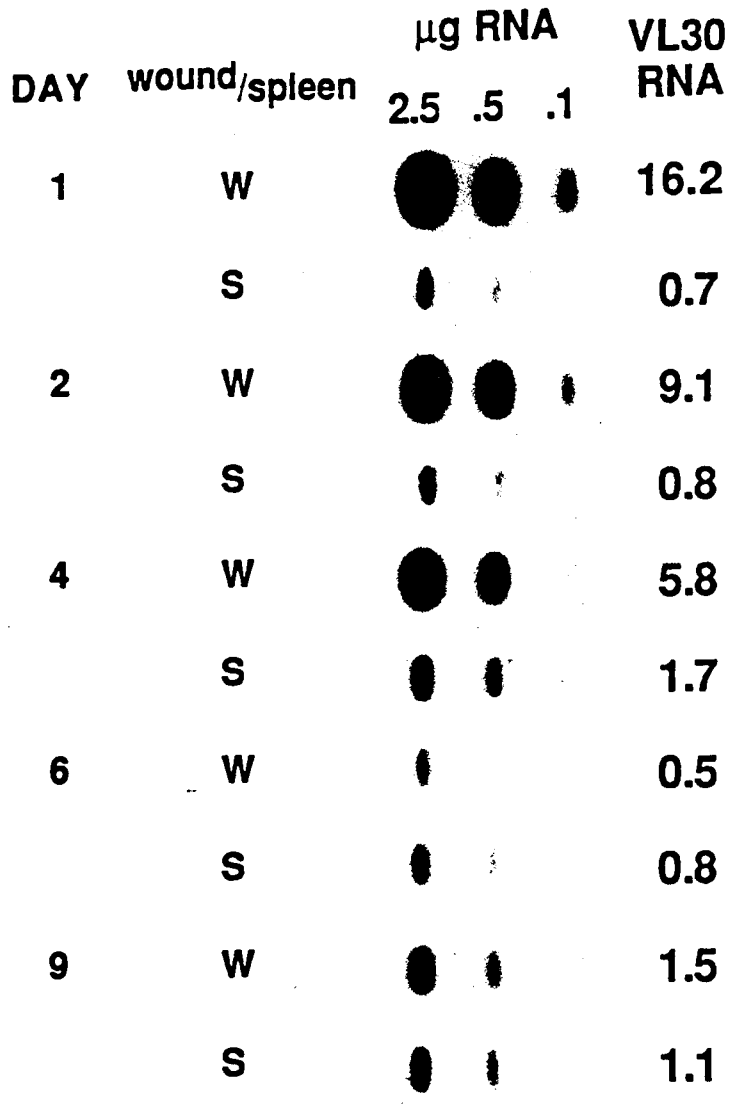


FIG. 2. The p34/lactate dehydrogenase-k anoxic response protein is associated with wound healing, being expressed within the granulation tissue and later found circulating in the vascular network. (A) The p34 protein is induced in fibroblasts between eight and sixteen hours into their exposure to anoxia. FRE rat fibroblast extracts were assayed for the p34/LDH-k by nondenaturing gel electrophoresis and activity staining, as described [18]. (B) The p34 anoxic response protein is found at its maximal level in wound granulation on the second day. 10 μg of protein extract from polyvinyl alcohol sponge pieces, implanted as in figure 1, were electrophoretically analyzed and activity stained. (C) The p34 protein is also detectable circulating in human patient sera, where it is found one to two weeks postoperatively. Data is from representative human patients undergoing colorectal surgery; similar results are seen with cancer and non-cancer patients. 100 microliters of sera were electrophoretically analyzed as in [32].

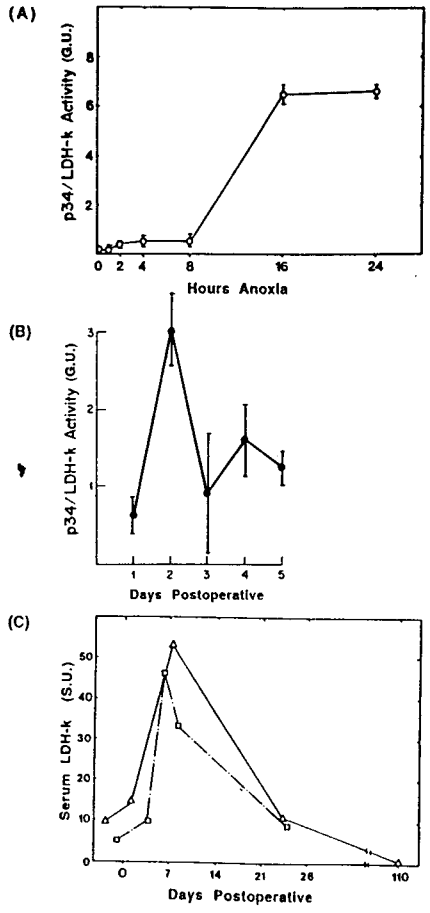


FIG. 3. Secretory proteases are induced in fibroblasts by anoxia, as detected by zymography. FRE cells were placed in serum free media immediately prior to being placed in the anoxic culture incubator. At the indicated time points culture dishes were removed and the media collected. Cells were also lysed in 0.5M Tris pH 6.8, 2% SDS and 20% glycerol; total cell protein determined by the modified Bradford assay. Media was dialyzed, lyophilized, and electrophoresed on 10% SDS PAGE, in 0.75 mm thick gels which also contained 0.1% gelatin. Media corresponding to 190 μg of intracellular protein was analyzed in each lane. At the completion of electrophoresis, gels were soaked for 30 minutes at room temperature in 100 ml of 2.5 per cent triton X-100, followed by 30 minutes in 100 ml of 50 mM Tris pH 7.5, 0.2M NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35, as per Brown et al.
[33]. Protease activity was developed overnight in 100 ml of the latter buffer, with incubation at 37°. Gels were then stained for protein with coomassie blue R250 and destained extensively, all in 50 per cent methanol and 7.5 per cent acetic acid.

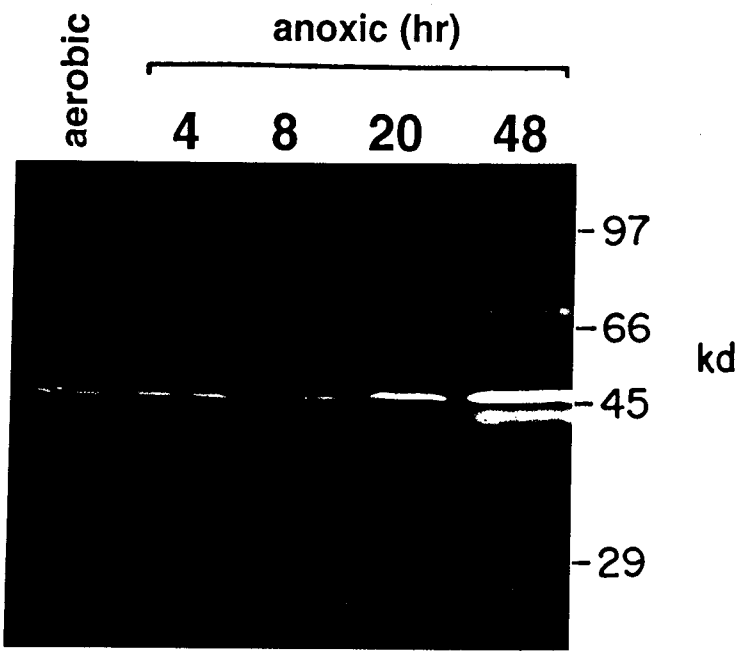


FIG. 4. The 51-kilodalton protein secreted by anoxic fibroblasts is procathepsin D.
(A) The 51 kd secretory protein comigrates on SDS-PAGE with one of three forms of procathepsin D. Lane [1] ^{14}C molecular weight standards, [2] proteins secreted by aerobic FRE fibroblasts, [3] proteins secreted by FRE fibroblasts after 16 hours of anoxia, [4] intracellular extract of anoxic FRE cells, immunoprecipitated with preimmune sera, [5] intracellular extract of aerobic FRE cells, immunoprecipitated with polyclonal rabbit antisera to cathepsin D, [6] intracellular extract of anoxic FRE cells, immunoprecipitated with anti-cathepsin D.
(B) Secretory proteins produced by anoxic FRE cells contain a protein immunoprecipitated by anti-cathepsin D. Secretory proteins from aerobic or 16 hour anoxic FRE cells were reacted with preimmune sera or polyclonal anti-cathepsin D, and the resulting immunoprecipitates analyed by SDS-PAGE.
(C) Cleveland gel analysis of the p51 proteins secreted by anoxic fibroblasts shows extensive similarity to the p51 procathepsin D immunoprecipitated from intracellular extracts. Lane 1, input secretory p51; lanes 2 and 3, immunoprecipitated p51 cleaved with 0.1 and 0.2 µg V-8 protease; lanes 4 and 5, secretory p51 cleaved with 0.1 and 0.2 µg V-8 protease.

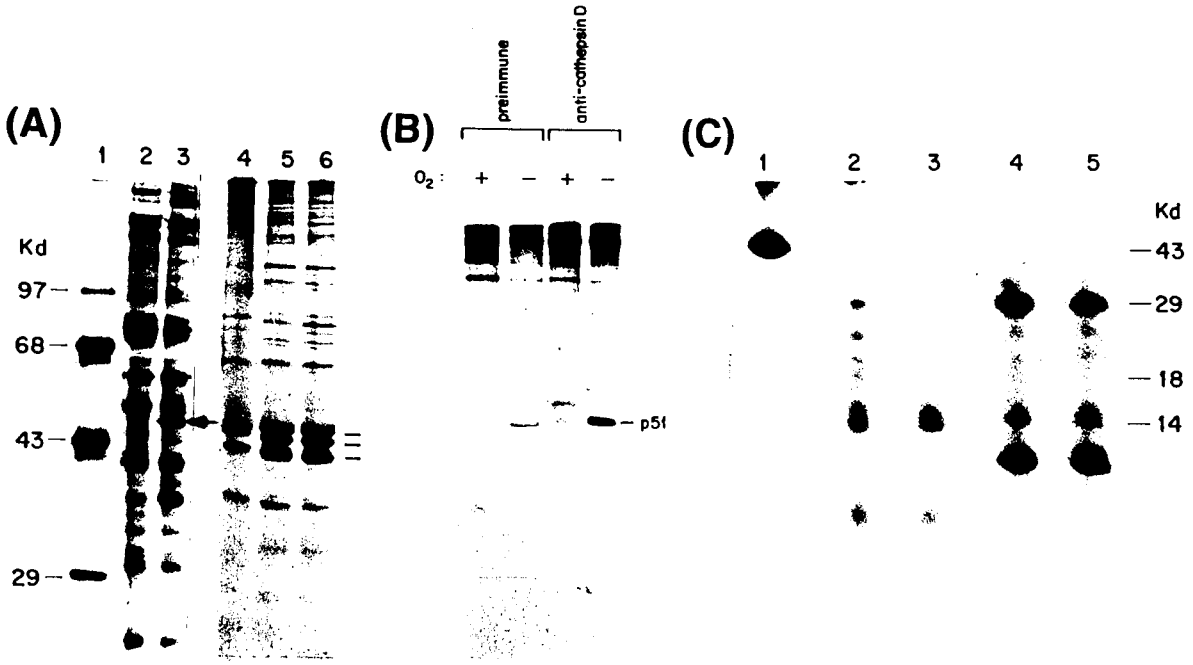


FIG. 5. Secretion of procathepsin by anoxic fibroblasts reflects processes at the posttranslational level.
(A) Slot blot analyses indicate equivalent transcription of cathepsin D mRNA occurs in aerobic and 16-hour anoxic fibroblasts. In contrast, the S-class of VL30 elements, which is also strongly induced within healing wounds (Fig. 1), is induced over 50-fold by anoxia.
(B) Secretion of procathepsin D may reflect rerouting effected by two proteins found associated with procathepsin D in immune precipitates of anoxic cell extracts. These proteins, of 78 and 74 kd, evidently do not represent cross-reacting proteins since they are not seen in immune precipitates of boiled extracts of anoxic fibroblasts. ^{35}S met labeling and electrophoretic analyses were as in fig. 4.

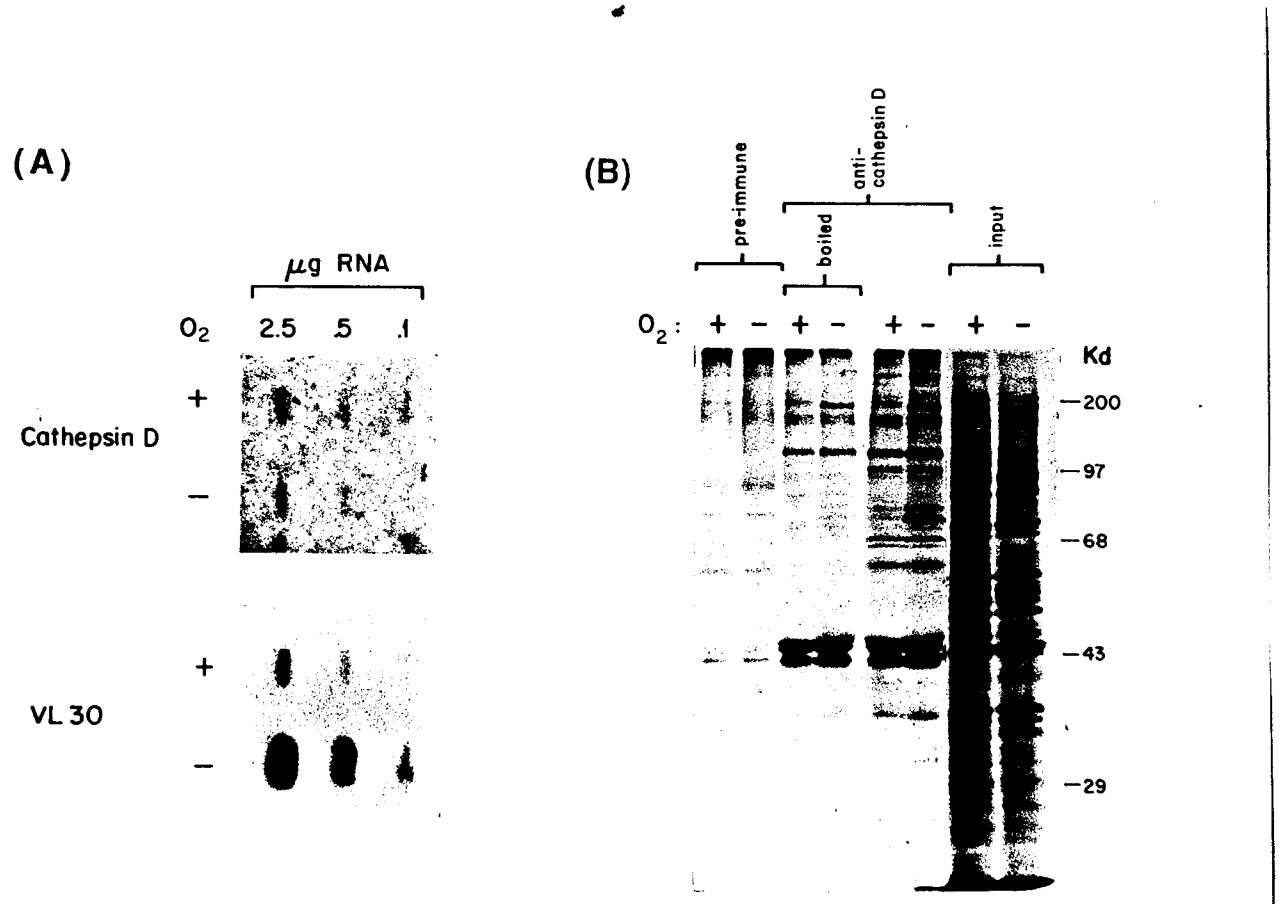


FIG. 6. Shifts in the fibroblast secretory protein profile occur after return to aerobic conditions, including secretion of procollagen. Secretory proteins produced by FRE fibroblasts were labeled with ^{35}S methionine and processed, as described [1]. 10 cm dishes were plated with 10^6 cells 24 hours prior to being placed under an anaerobic atmosphere for 44 hours, and secretory proteins were collected at the indicated time points. Cell cultures were media changed at days one and four of aerobic recovery. Secretory proteins were analyzed on 10% SDS gels, with 2 X 10^4 cpm of acid insoluble material loaded in each lane.

Lane	Sample
1	secretory proteins produced from 40-44 hours into anoxia; labeled while still anoxic.
2	secretory proteins produced immediately after return to aerobic conditions.
3	aerobic recovery, 1 day.
4	aerobic recovery, 2 days.
5	aerobic recovery, 3 days.
6	aerobic recovery, 7 days.
7	aerobic recovery, 9 days.
8	continuous aerobic, day 0 after plating.
9	continuous aerobic, day 2.
10	continuous aerobic, day 4.
11	continuous aerobic, day 9.

The uppermost major secretory protein produced by anoxic recovery and continuous aerobic cells was found to be immunoprecipitated by polyclonal rabbit antisera to rat collagen, type-I, and comigrated with procollagen (data not shown).

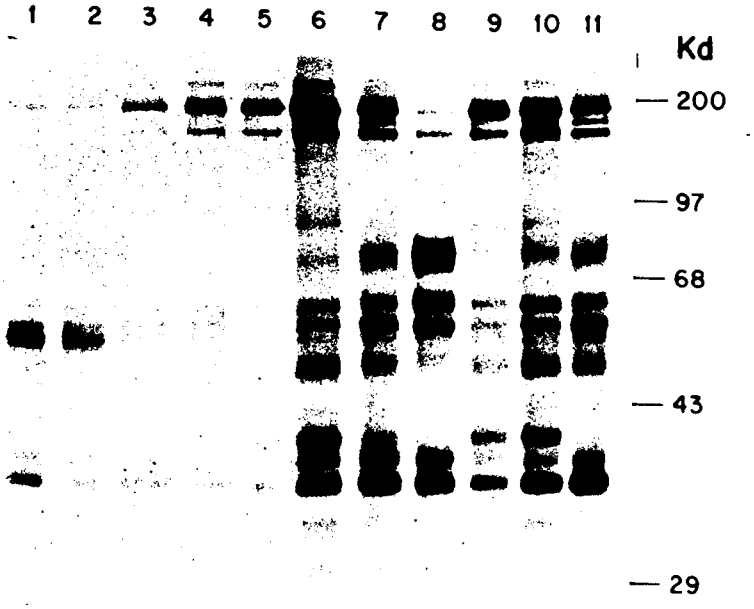


FIG. 7. A contractile response is induced in fibroblasts approximately one week after exposure to anoxia. This effect is induced by a minimal amount of anoxia between eight and sixteen hours. FRE fibroblasts at 10^6 cells per 10 cm dish were cultured anoxically for the indicated time periods, returned to aerobic conditions without media changing, and photographed under phase microscopy 7 days later.

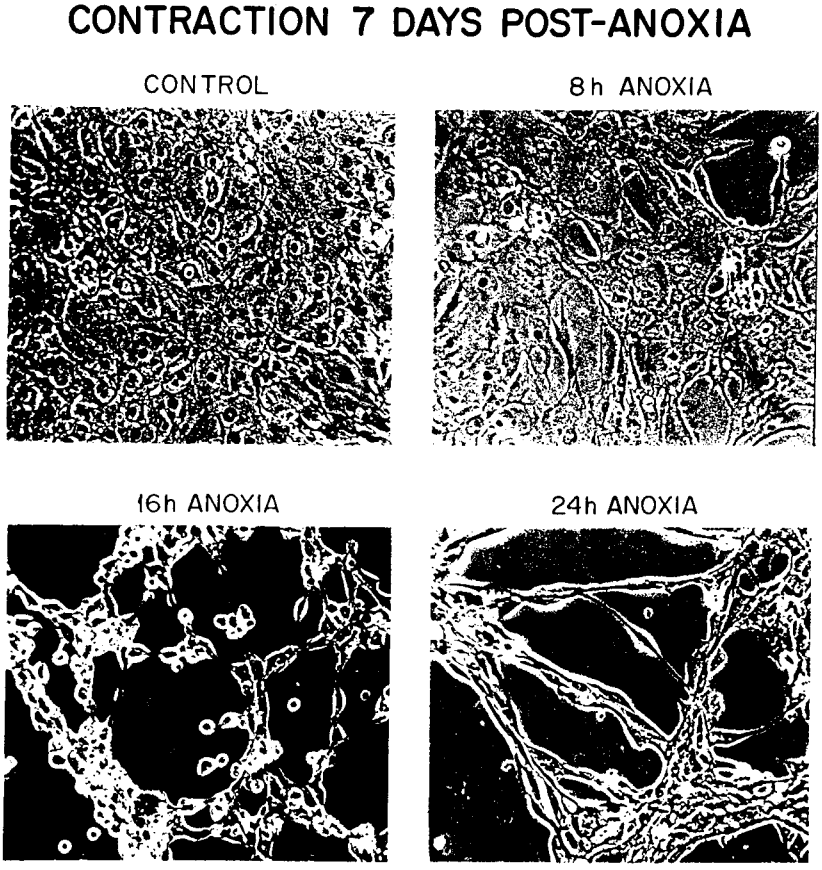
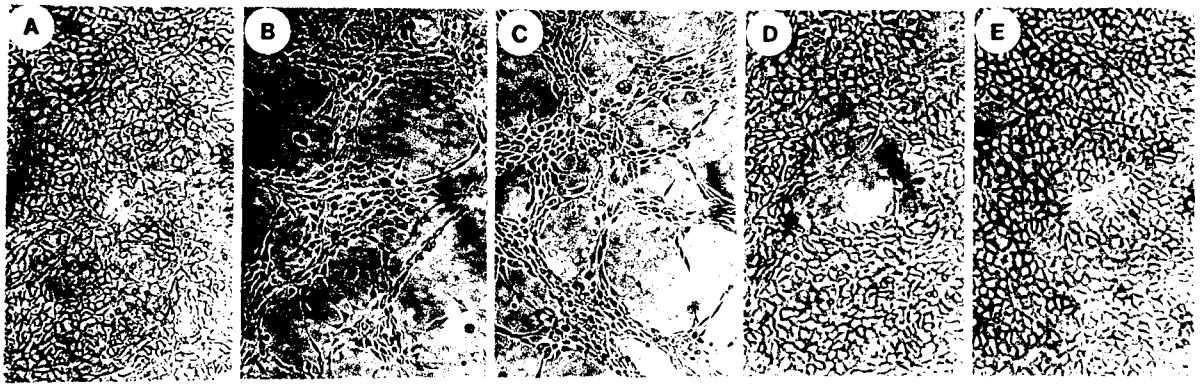


FIG. 8. The anoxia inducible contractile response is mediated by a factor secreted during the first two days after return to aerobic conditions. FRE cells were cultured under anoxic conditions for two days, returned to aerobic culture, and then media was harvested at the indicated time points. FRE cells which had never been anoxic were assayed for contraction-inducing factor by being treated with this media for a period of seven days. Media being assayed was from: A, immediately after return to aerobic conditions; B, 1 day; C, 2 days; D, 3 days, E, 4 days. With additional culture in this factor containing media, the cells shown in panels B and C progressed to fully contract as in Fig. 7.



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