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Effect of β-Aminopropionitrile and Ascorbate on Fibroblast Migration (42745)

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Abstract. Ascorbate and β-aminopropionitrile (BAPN) have direct, but diverse affects on collagen matrix production. Ascorbate is necessary for the intracellular hydroxylation of prolyl and lysyl residues during collagen biosynthesis whereas BAPN inhibits the enzyme lysyl oxidase in the extracellular space thus preventing collagen crosslink formation. To study the influence of these two agents on fibroplasia, an *in vitro* model was used to analyze fibroblast migration, proliferation, and collagen synthesis. Biopsies of chicken tendon were covered with a fibrin clot to simulate an *in vivo* wound environment, and then they were exposed to either ascorbate or BAPN for up to 7 days. Fibroblast migration into the fibrin clot was measured using a Zeiss Mopp II planimeter, DNA synthesis by ^{125}IUDR incorporation, and collagen synthesis by [^{3}H]proline incorporation into collagenase-digestible protein. Tendon biopsies treated daily with fresh ascorbate (0.1 m*M*) had significantly greater fibroblast migration than controls without ascorbate ($P < 0.05$). Cellular proliferation, collagen synthesis, and total protein synthesis were not significantly altered by ascorbate treatment. In contrast, BAPN inhibited fibroblast migration in a dose-dependent fashion without inhibiting proliferation (0.25 and 0.5 m*M*), collagen, and noncollagen protein synthesis. Therefore, the effect of BAPN on migration does not appear to be due to generalized cytotoxicity. These combined studies suggest that compounds such as ascorbate and BAPN which can modify collagen may also modify fibroblast migration.

The nature of the cellular milieu and surrounding collagen matrix at a wound site are critical mediators of fibroplasia. The process of fibroplasia is a combination of fibroblast proliferation, migration, and collagen formation (1, 2). Modulation of any one of these events could theoretically alter fibroplasia and thus may provide a clinical means for controlling the wound healing process. An *in vitro* model of fibroplasia was previously developed in our laboratory to study simultaneously fibroblast migration, proliferation, collagen, and noncollagen protein synthesis (3). This model allows analysis of the fibroplasia system as a unit, and also allows one to study the isolated effects of biochemical mediators upon fibroplasia.

To further define the importance of the collagen matrix in fibroplasia, two well-defined collagen modulators, ascorbate and β-aminopropionitrile (BAPN), were studied in the *in vitro* fibroplasia model. Ascorbate is a cofactor needed for the hydroxylation of prolyl (4) and lysyl (5) residues during collagen synthesis. In its absence, adequately hydroxylated collagen is not readily secreted from the golgi of the fibroblast (2). Therefore, the extracellular matrix is abnormal and wound healing is impaired (6–8). Furthermore, the total amount of matrix is reduced because underhydroxylated collagen is thermally less stable (9, 10) and more rapidly degraded (11). In addition, a fully hydroxylated collagen molecule is a better substrate for initial crosslink formation (12). Ascorbate has also been shown to potentiate the maximum expression of the collagen gene (13–15). Therefore, ascorbate can affect directly the total amount of collagen produced and can affect indirectly the degree of cross linked collagen available.

The lathyrogen, BAPN, interferes with collagen crosslinking by irreversible inhibition of lysyl oxidase (16–18). This enzyme is necessary for the oxidative deamination of lysyl and hydroxylysyl residues in the collagen molecule (19). Following deamination, there is the formation of reactive aldehyde moieties that undergo aldol condensation to form stable crosslinks between collagen molecules. Since BAPN blocks the collagen crosslinking step, it provides another mecha-

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nism with which to study the role of maturation of the extracellular matrix during fibroplasia.

Materials and Methods. Flexor digitorium profundus tendons of white Leghorn chickens (2.5 to 3 kg) were harvested as described previously using sterile conditions and rinsed in Dulbecco's modified Eagle's medium (DMEM) (3). Biopsies were excised using sterile 2-mm trephines and placed in multiwell culture dishes (Costar No. 3524, Cambridge, MA). They were covered initially with 5 μl of thrombin solution (2 mg/ml in DMEM), and then covered by 50 μl fibrinogen solution (4 mg/ml KABI grade G, Stockholm, Sweden). Plates with biopsies were placed in a humidified incubator at 37°C for 1 hr to allow for maturation of the fibrin clot and then the wells were filled with 1 ml of medium.

The first experiments consisted of two groups. Group 1 was incubated with DMEM containing 10% fetal calf serum (DMEM-10) and penicillin with streptomycin. Group 2 was incubated with the same medium as group 1 with the addition of 0.1 m*M* ascorbate. The addition of 0.1 m*M* ascorbate is known to provide adequate cofactor for hydroxylation without any cellular toxicity (20). All biopsies were replenished with 1 ml of medium daily since the active half-life of ascorbate is approximately 12 hr (21). Culture medium containing 10% fetal calf serum was used to provide a maximally stimulated environment, similar to that found in a wound site *in vivo* (3).

A second set of experiments was designed to test the effect of inhibition of collagen crosslink formation on fibroplasia. Biopsies were incubated with 1 ml of DMEM-10, containing 0.1 m*M* ascorbate and with 0.25, 0.5, 1.0 m*M*, or no β-aminopropionitrile (BAPN-fumarate, Aldrich Chemical Co., Inc., Milwaukee, WI). The culture medium covering the biopsies was replenished daily with fresh medium.

Fibroblast migration. Fibroblast migration into the fibrin matrix was quantitated using an area subtraction technique as described previously (3). A projection microscope was used, and a permanent record of the tendon biopsy and the area of fibroblast migration was recorded. The area of fibroblast migration was then calculated using a Zeiss Mopp II planimeter by subtracting the tendon biopsy area from the total area of fibroblast migration. Migration data were obtained on the fifth day of incubation unless indicated otherwise.

Fibroblast proliferation. To quantitate DNA synthesis, the biopsy cultures were incubated for 12 hr with 1 μCi/ml ^{125}IUDR (2000 Ci/m*M*, New England Nuclear, Boston, MA). ^{125}IUDR is a pyrimidine analog and is incorporated into DNA (22–24). To increase ^{125}IUDR incorporation, 10^{-5} *M*, 5-fluorodeoxyuridine (FUDR), which inhibits endogenous thymidine synthesis (25), was also added during the time of isotope pulsing. Cytosine arabinoside (0.5 μg/ml), a specific inhibitor of DNA synthesis (26), was added to a parallel set of biopsies to assess background radioactivity. Once incubation with isotope was completed, the biopsies were rinsed three times with cold phosphate-buffered saline (PBS). The contents of the individual culture wells were dissolved in 1 *N* sodium hydroxide (NaOH) at 37°C for 24 hr. The NaOH destroys nucleases, hydrolyzes RNA, and solubilizes the tendon biopsy (27–29). The NaOH was neutralized with hydrochloric acid and the DNA was precipitated at 4°C by the addition of trichloroacetic acid (TCA) to give a final concentration of 5% TCA. Microstir bars were utilized to mix the precipitates while being washed three times with 5% TCA. After the final wash, precipitates were dried and sealed in the multiwell plates with clear acrylic spray. Individual culture wells were numbered, separated with a band saw, and placed in counting vials, and the radioactivity was measured using a Beckman gamma scintillation counter (23).

Collagen and noncollagen protein synthesis. To quantitate collagen and noncollagen protein synthesis, biopsies were incubated for 12 hr with medium supplemented with [^{3}H]proline (20 μCi/ml, 20 Ci/m*M*, Amersham International, Amersham, UK). Cyclohexamide (2.8 μg/ml) was added to control incubations to inhibit protein synthesis and thus determine background radioactivity.

The biopsy cultures were washed three times with cold PBS, and then heated to

90°C in a water bath for 10 min to inactivate proteases. Once cooled, the samples were sonicated to release all intra- and extracellular proteins from the tendon-clot matrix. The samples were treated for 5 min at 37°C with RNAse (20 μg/ml) to destroy residual tRNA-[^{3}H]proline complexes.

The samples were precipitated at 4°C with TCA to achieve a concentration of 5%. Three additional washes with 5% TCA at 4°C were performed and then the precipitates were dissolved in 0.6 ml of 0.2 *N* NaOH. The radioactivity in collagen was released by digestion with purified bacterial collagenase as described previously (30). Briefly, samples contained 0.2 ml of the aliquot to which 0.3 ml of a cocktail of 0.1 ml 1.0 *M* Hepes buffer (pH 7.2), 0.01 ml 25 m*M* $CaCl_2$, 0.02 ml *N*-ethylmaleimide (NEM, 7.82 mg/ml in H_2O), and 0.17 ml deionized water. The pH of the samples was adjusted to 7.2 and each 0.5-ml sample received either 10 μl of enzyme buffer to serve as an incubation blank or 10 μl of bacterial collagenase. Incubation at 37°C for 90 min allows for complete digestion of the collagen (30). Samples were placed on ice and the undigested noncollagen proteins precipitated with 0.5 ml of 10% TCA–0.5% tannic acid. Following centrifugation at 400*g* for 10 min, the respective supernates were placed in positive- or negative-labeled vials indicating the addition of collagenase or enzyme buffer. The pellets were resuspended in 0.5 ml of 5% TCA–0.25% tannic acid and centrifuged again and the supernates were added to the respective vials. The protein precipitates from the samples receiving collagenase were resuspended in a total volume of 1.5 ml of 5% TCA–0.25% tannic acid and transferred to separate scintillation vials. Triton–Liquifluor (1:2) was added to each vial and the radioactivity was measured using a Beckman scintillation spectrometer. This procedure for measuring collagen synthesis has been shown to release greater than 91% of the total newly synthesized hydroxyproline in connective tissue (31).

Results. *Effect of ascorbate on tendon fibroplasia.* Incubation of the tendon biopsy cultures in medium replenished with fresh ascorbate (0.1 m*M*) each day showed a significantly greater migration of fibroblasts into the surrounding fibrin clot on Days 5 through 7 compared to control cultures incubated in the absence of ascorbate (Fig. 1). Beyond 7 days, the fibrin–collagen matrices in the ascorbate-treated cultures were so densely populated with fibroblasts that they began to contract from the surface of the culture plates and coalesced with the tendon explant. In a separate experiment, a larger number of cultures were examined ($n = 26$), and those explants incubated continuously for 5 days in the presence of fresh ascorbate had an area of fibroblast migration that was 133% compared to the control culture area of migration ($P < 0.05$, data not shown).

One possible explanation for enhanced fibroblast migration in the presence of ascorbate would be increased cell proliferation. Therefore, on Day 5, cultures treated with or without ascorbate were pulse-labeled with ^{125}IUDR to measure fibroblast proliferation. However, the data in Fig. 2 show there was no significant difference in the total amount of ^{125}IUDR incorporated into the DNA of the ascorbate-treated explants compared to the controls.

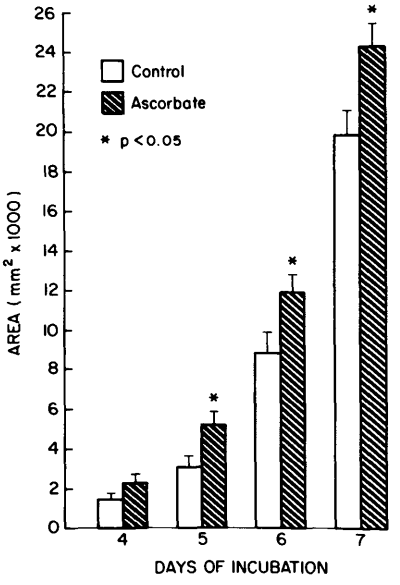

FIG. 1. The effect of ascorbate on tendon fibroblast migration on Days 4 through 7. The area on the graph represents the magnified, projected area that was actually quantified. The values represent the means ± SEM obtained from 16 observations for each group.

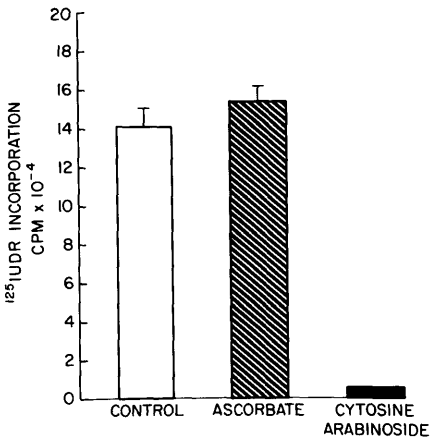

FIG. 2. The effect of ascorbate on tendon fibroblast proliferation. Tendon biopsy cultures were maintained in either the presence or absence of ascorbate for 5 days. On the fifth day, fibroblast proliferation was measured by quantifying ^{125}IUDR incorporation into DNA. Background radioactivity was determined by incubating a parallel set of cultures with cytosine arabinoside during the incubation with the isotope. The values represent the means ± SEM obtained from 11 observations for each group.

It is possible that ascorbate could stimulate collagen production and thus enhance fibroblast migration onto the new matrix. However, in this particular biopsy culture system and in the presence of 10% fetal calf serum, the addition of ascorbate did not augment collagen synthesis (Table I) as has been observed in other studies of isolated fibroblast cultures (13–15). Therefore, since ascorbate did not increase fibroblast proliferation and collagen synthesis, these mechanisms do not appear to be the explanation for enhanced movement of the fibroblasts from the tendon biopsy into the surrounding fibrin matrix.

Effect of BAPN on tendon fibroplasia. The second objective of this investigation was to determine the importance of a crosslinked collagen matrix on fibroplasia. Exposure of the tendon explants to increasing concentrations of the collagen crosslinking inhibitor β-aminopropionitrile resulted in inhibition of fibroblast migration (Fig. 3). There was a dose-dependent inhibition of migration from the lowest concentration of 0.25 m*M* BAPN to the highest concentration of 1.0 m*M* (Fig. 3).

Once again the question arose as to whether the inhibitory effect of BAPN was due to a direct action on the process of cell migration or was due to some other function of the cell. To answer this question, the effect of BAPN on cell proliferation was next examined. Treatment of the tendon explants with increasing concentrations of BAPN resulted in a "bell-shaped" response curve. There was a significant stimulation of DNA synthesis at the lowest concentration tested (0.25 m*M*; $P < 0.05$), no effect at 0.5 m*M*, and a decrease at 1.0 m*M* (Fig. 4). When parallel cultures were incubated with BAPN, there was no significant effect on either net collagen or noncollagen protein synthesis (Fig. 5).

Discussion. These studies were designed to examine the biological effect of two well-characterized modulators of collagen synthesis and collagen matrix formation to gain a better understanding as to how these agents may function in the *in vivo* wound healing environment. Therefore, an *in vitro* model of

TABLE I. EFFECT OF ASCORBATE ON TENDON FIBROBLAST COLLAGEN SYNTHESIS

	Collagen (cpm/sample)	Noncollagen protein (cpm/sample)	Percentage collagen synthesis (%)
Control (10)	1201 ± 39	1033 ± 71	18.2 ± 1.1
Ascorbate treated (11)	1240 ± 88	897 ± 92	21.6 ± 1.9

Note. After 5 days of incubation, with or without daily feeding with medium containing fresh ascorbate (0.1 m*M*), the culture medium was removed and replaced with fresh DMEM, minus serum and containing [^{3}H]proline (20 μCi/ml), with or without ascorbate. After 12 hr of additional incubation, the culture medium and the biopsies were removed and the combined samples were analyzed for isotope incorporation into collagen and noncollagen protein (30). Relative collagen synthesis (%) was calculated using a formula to correct for the enriched imino acid content of collagen (36). Data are expressed as means ± SEM; number of observations in parentheses.

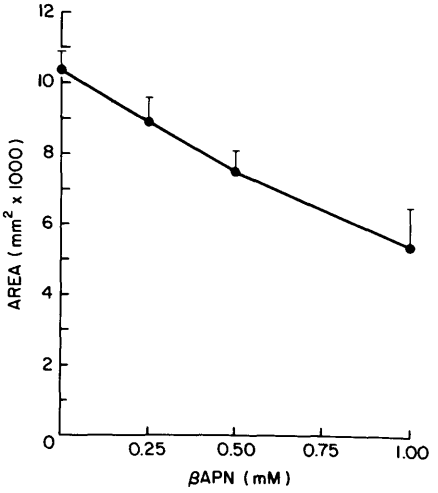

FIG. 3. The effect of BAPN on tendon fibroblast migration. Tendon biopsy cultures were incubated with the indicated concentration of BAPN for 5 days and then the area of fibroblast migration was measured. The area on the graph represents the magnified, projected area that was actually quantified. The values represent the means ± SEM obtained from 12 observations for each data point.

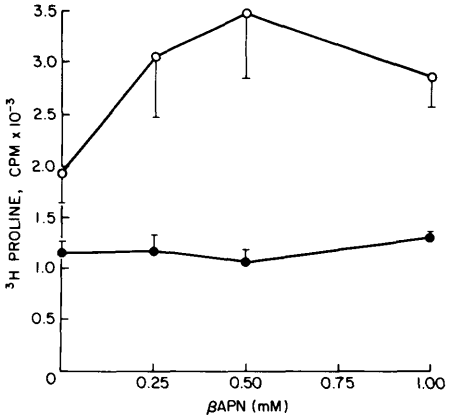

FIG. 5. The effect of BAPN on tendon fibroblast collagen and noncollagen protein synthesis. Parallel tendon biopsy cultures were incubated with the indicated concentration of BAPN for 5 days and then exposed to [^{3}H]proline to measure isotope incorporation into newly synthesized collagen (closed circles) and noncollagen protein (open circles) (30). The values represent the means ± SEM for each determination. The numbers of observations for each data point were as follows: control, 6; 0.25 m*M* BAPN, 7; 0.5 m*M* BAPN, 6; and 1.0 m*M* BAPN, 5.

fibroplasia was chosen to study the effects of ascorbate and β-aminopropionitrile on fibroblast proliferation, migration, and collagen synthesis. The advantage of this model is that all three parameters of fibroplasia can be analyzed simultaneously under the exact same conditions of incubation. It is assumed that since the migrating fibroblasts are the progeny of the fibroblasts within the biopsy proper, that both populations exhibit the same functional characteristics.

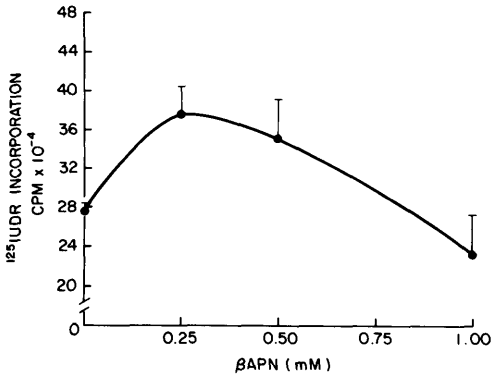

FIG. 4. The effect of BAPN on tendon fibroblast proliferation. The same tendon biopsy cultures used for determining the effect of BAPN on fibroblast migration (Fig. 3), were then incubated with ^{125}IUDR to measure DNA synthesis on Day 5. The values represent the means ± SEM for each determination. The numbers of observations were as follows: control, 6; 0.25 m*M* BAPN, 6; 0.5 m*M* BAPN, 5; and 1.0 m*M* BAPN, 5.

In the first study, it was found that continuous exposure of the tendon explants to ascorbate resulted in a significant stimulation of fibroblast migration. This specific response of increased migration was independent of an effect of ascorbate on fibroblast proliferation or on collagen synthesis. It was surprising that ascorbate did not alter collagen synthesis in this system as has been observed in other studies (13–15). The explanation may be that the present studies employed tissue explants rather than isolated fibroblasts in culture. In addition, the present studies were carried out in the presence of 10% fetal calf serum, a condition which can modify the effect of ascorbate on collagen synthesis (32). These findings would suggest that the collagen produced in the presence of ascorbate is more completely hydroxylated and, as such, serves as a better matrix for

fibroblast migration. It is well documented that ascorbate enhances collagen synthesis (13–15) and hydroxylation (4, 5), but the present observations suggest that a fully hydroxylated collagen matrix may provide a surface which is optimal for cell movement. If this hypothesis is indeed true, there may be important clinical implications whereby movement of fibroblasts into the wound site may be enhanced by ascorbate. Leukocytes trapped in the initial blood clot that forms in the wound are a rich source of ascorbate (33) and thus provide a ready supply of this essential vitamin for optimal matrix formation. It is also possible that fibroblasts may be attracted to the source of ascorbate by a process similar to chemotaxis.

In the second series of the studies, it was observed that the collagen crosslink inhibiting agent, β-aminopropionitrile, caused a dose-dependent inhibition of fibroblast migration. This inhibitory effect was not explained by a generalized toxic effect of BAPN on the fibroblasts because there was no inhibition of DNA synthesis observed at 0.25 and 0.5 m*M* BAPN. Likewise, BAPN did not inhibit collagen or noncollagen protein synthesis. Therefore, these studies suggest that collagen crosslink formation may be important for cell attachment and movement.

Translating this basic information to the wound environment would imply that a mature, fully crosslinked collagen matrix is required for fibroblast movement into the wound area. These present observations are consistent with reports that BAPN treatment results in a reduced number of fibroblasts in the wound site (34, 35). Although it is not generally considered that collagen crosslink formation is a rate-limiting step in normal wound healing, perhaps agents which promote or allow optimal crosslink formation may enhance the overall healing response. In conclusion, these studies suggest that agents which are known to modify collagen may also modify fibroblast migration.

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