INVESTIGATION OF COMPARATIVE PHARMACOLOGICAL RESPONSIVENESS OF RAT SUBCUTANEOUS FASCIA AND EXCISIONAL WOUND GRANULATION TISSUES

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ABSTRACT

In the present *in vitro* study, using superfusion technique, the pharmacological responsiveness of the excisional wound granulation tissue myofibroblasts to a variety of agents were compared with those of normal connective tissue taken from the superficial fascia. Bolus doses of angiotensin II (0.1 to 1000nM), mepyramine (1 to 128μM), calcium chloride (75 to 300μM) and potassium chloride (100 to 400μM) were added to the superfusate solution and the responses were recorded on Narco chart recorder. The results showed that both types of tissues have qualitatively similar characteristic responsiveness to potassium and calcium ions, mepyramine and angiotensin II. However, quantitatively, they were distinctly different, the myofibroblasts from granulation tissues produced greater contractile responses to mepyramine and angiotensin II, whilst they lost most of their responsiveness to potassium and calcium ions. The present study demonstrated that these tissues respond to pharmacological manipulation, but the responses they produce are distinct from smooth muscle cells and need to be considered further in experiments involving wound healing.

Keywords: Myofibroblast, Excisional wound, Granulation tissue, Mepyramine, Angiotensin II, Potassium ion, Calcium ion, Rat

INTRODUCTION

Wound healing is an orchestrated phenomenon of complex interactions between many factors in the wound space leading to tissue remoulding and restoration of the integrity of the damaged tissues (1). In humans it is always accompanied by discernible scar formation (2). Among many integrated interactions in the early stages of wound repair it appears that the fibroblasts are recruited from neighbouring connective tissues (the fascia), where they proliferate and differentiate into active contractile myofibroblasts (3). Once the tissue integrity is restored, the myofibroblasts disappear, leaving an acellular fibrous collagen scar filling the damaged area (4). However, in abnormal conditions such as hypertrophic scar or during Dupuytren's contracture their presence persist and produce consistent contraction (5, 6).

The transient appearance of the myofibroblasts during wound healing has been documented (4) but factors regulating their activity, especially those involved in wound closure are not well understood. The existence of different phenol-types of myofibroblasts has been reported (2). On the other hand, the important and less understood aspect is the pharmacological characteristics of wound granulation tissue. Furthermore, a few studies with the view point of pharmacological responsiveness to a variety of agonists that were undertaken in the last three decades show conflicting results. They used different tissue sources and different experimental protocols, ranging from different recording techniques, physiological solution and even different preloading tensions and temperatures (3, 7-12). To clarify some aspects of these discrepancies, the present study, therefore, was designed to determine whether rodent granulation tissue myofibroblasts respond similarly to normal superficial fascia fibroblasts from which they are derived. For this purpose, by use of an *in vitro* superfusion technique, the responses of rat normal subcutaneous fascia and excisional wound granulation tissues to some drugs such as mepyramine, a potent H_1 antagonist and an agent found to selectively to induce contraction in these tissues (13, 14), angiotensin II, potassium and calcium chloride were detrmined and compared (15, 16).

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MATERIAL AND METHODS

Materials

Isolated strips of the loose subcutaneous connective tissues taken from eight male Hooded Lister rats (Bradford University strain) with the body weight range of 250-350g were used in this study. All rats were housed in plastic, clean sawdust floored, cages (59x38x22 cm) in groups of 5 and they had free access to water and standard food pellets (CRM-P-Special Diet Services, Witham, Essex, UK). All drugs and reagents employed in this study were purchased from Sigma (UK).

Preparation of the strips of the loose connective

Samples of the superficial fascia were always selected from the lower dorsal site after sacrificing each animal with an intraperitoneally administered dose of sodium pentobarbital (200 mg/Kg) and cervical dislocation. By use of a fine forceps, a thin piece of the exposed superficial fascia was pulled gently from the underlying adipose tissue of the skin and by the aid of a blunt-ended scissors, a small incision was made from the edges extending about 3cm down the curvature of the body. A fat free, thin transparent strip of 1x3cm was removed and immediately placed in Krebs Henseleit solution. The resulting tissue strips (1 x 2 cm) were mounted under 2g isometric tension for experimental purposes.

Preparation of strips of excisional wound granulation tissue

The method reported by Cross and Naylor was used in this study [14]. Basically, eight rats were anaesthetised with isoflurane (using O_2 and N_2O as carriers) and the lower left flank was clipped with electric clipper and wet shaved with a scalpel blade. After disinfecting with alcoholic chlorhexidine gluconate solution, it was allowed to dry, and the template (15x15mm) was placed on the site, and the outline was traced with a fine felt-tipped pen. The median border of the template was oriented parallel with, about 3cm from, the sagittal axis of the animal. A full thickness wound was made by excising the skin within the confines of the traced area down to the level of the loose subcutaneous connective tissue. Care was taken to ensure that wound edges were sharply defined and most of the underlying connective tissues remained in the wound bed, by the use of a sharp size 15 scalpel blade. No dressings was applied to the wound site and each animal was placed in a separate cage and allowed to recover from anaesthesia before being returned to the holding room, and had free access to food and water.

At the end of the seventh day, animals were sacrificed by an intraperitoneally administered dose of sodium pentobarbital (200mg/Kg) and cervical dislocation. The granulation wound tissue together with about 0.3mm from the normal surrounding skin edges were removed. Longitudinal strips of the granulation tissue were cut, were held by a cotton thread, tied vertically and suspended under 2g isometric tension in the path of the superfusate solution [17].

Pharmacological investigations

The following drugs were used: Angiotensin II (0.1 to 1000 nM), mepyramine (1 to 128μM), calcium chloride (75 to 300μM) and potassium chloride (100 to 400μM) as bolus doses. The volume range administered varied from 32 to maximum 128μl. In addition, in order to rule out the vehicle effects, acidified or alkaline water having equal volume and pH to drugs solutions were added to the superfusate.

Statistical Evolution

The data were analysed using ANOVA, followed by Dunnet's test and $P<0.05$ was considered as the level of significance.

RESULTS

Responses to mepyramine

Responses to mepyramine (1 to 64μM) in wound granulation tissues and superficial fascia showed that the magnitude of the responses to mepyramine were significantly greater in wound granulation tissues in comparison with superficial fascia (Table 1). The threshold responses in wound granulation tissues were initiated at a lower concentration. While mepyramine at concentration of 1μM induced a contractile response in wound granulation tissues, for comparable responses in superficial fascia a concentration of 16M of mepyramine was required (Table 1). The responses to 64μM mepyramine were 70% greater in wound granulation tissues compared to superficial fascia ($P<0.001$), using superfusion flow rate of 3 ml/min. The mean duration of wound granulation tissues response to maximum dose of 16 μM was 10 minutes compared to a mean of 2 minutes in superficial fascia ($P<0.001$).

Responses to angiotensin II

The contractile responses in granulation tissues from the excisional wounds were found to be significantly greater than superficial fascia. The

Table 1: Summary of responses of the superficial subcutaneous fascia (SF) and wound granulation tissue (WGT) following bolus (doses) of mepyramine (MPY), angiotensin II (ANG), calcium chloride ($CaCl_2$), and potassium chloride (KCl), all drugs were administered at superfusion rate of 3 ml/min .

MPY (μM)	SF	WGT	ANG (nM)	SF	WGT	$CaCl_2$ (μM)	SF	WGT	KCl (μM)	SF	WGT
1	0	22^a±1	0.05	0	0	75	75±5	15^a±3	100	-32±4	-14^a±2
2	0	37^a ±2	0.1	0	37^a ±4	150	79±9	30^a±4	200	-59±6	-28^a±3
4	0	45^a ±4	1	13±6	49^a ±9	300	155^c±13	57^a±9	300	-81±8	-49^a±5
8	0	51^a ±8	10	14±3	65^a ±9						
16	20±3	77^a ±9	100	16±5	70^a ±8						
32	31±3	78^a ±8									
64	50^b±3	85^a ±8									

^{a}P<0.001 between WGT and SF for each of the drugs tested, ^{b}P<0.01 between responses of SF towards MPY and ANG, ^{c} P<0.01 between responses of SF to $CaCl_2$ relative to MPY and ANG, ANOVA followed by Dunnet's test. All figures are mean ± sem of n =8. – sign for potassium chloride represents relaxation responses.

threshold of activation of the contraction in granulation tissues from the excisional wounds were lower (0.05nM) than those for superficial fascia (0.1nM). The amplitude of the contractile responses in granulation tissue from excisional wounds were on average, more than 4 fold greater than those in superficial fascia (P<0.001) (Table 1). Furthermore, at all concentrations the duration of response to angiotensin II was on average 8 fold greater (P<0.001) in wound granulation tissue (mean 4 minutes) in comparison with those recorded for superficial fascia which had a mean durration of contraction of 30 seconds. The responses of superficial fascia to maximum dose of mepyramine compared to angiotenisin II were found significantly greater (P<0.01) (Table 1). This effect was not observed with wound granulation tissue.

Responses to potassium chloride

The relaxation responses in superficial fascia were similarly greater compared to the wound granulation tissue (Table 1). The relaxation responses were shown by a rapid initial phase followed by a gradual return to the original base line tension. By the use of high concentration of potassium chloride (400 μM) in superfecial fascia the maximum relaxation was observed within 30 seconds and the whole duration of the responses lasted approximately 2 minutes. Wound granulation tissue exhibited the same general profile as superficial fascia, that is a relaxation responses to potassium chloride. However, the responses in superficial fascia were 84% greater than wound granulation tissue (P<0.01).

Responses to calcium chloride

Calcium chloride (75 to 300μM) administered in bolus doses at a constant volume of 100μl induced a gradual contractile response, attaining maximal tension in about 1 minute in the superficial fascia which was followed by a gradual decline to the base line tension lasting on average 3 minutes. In contrast, the responses of wound granulation tissue were shallower and significantly lower (table 1) with a shorter (P<0.001) duration of contraction and an average duration of 1 minute. The mean of the contractile responses to 300 μM calcium chloride in the superficial fascia were 2.7 fold greater than that in the wound granulation tissue (P<0.001). Among the agents employed in this study, calcium chloride at maximum concentration of 300 μM produced greatest response of contraction in superficial fascia compared to other agents (P<0.01) (table 1)

DISCUSSION

Although elucidating the underlying mechanisms that govern the basis of the pharmacological responsiveness of both connective tissue and its various pathological forms, especially in coetaneous injury, is vital for scientifically based medical interventions during wound management, studies considering connective tissue pharmacology are conducted in only a limited number of centres throughout the world, and as a result makes this field of work drag behind other fields of pharmacological research. The first paper that appeared was in 1969 (18), and since then fewer than 15 papers have appeared in the literature.

In this study, it was demonstrated that the connective tissues are responsive to pharmacological manipulation. Furthermore, it was shown that the normal connective tissues taken from subcutaneous fascia and the granulation tissue prepared from excisional

wound have similar *pattern* of pharmacological responsiveness to a variety of drugs. This finding suggests that fibroblasts, the original cells from which myofibroblasts were evolved (19) retained some of their pharmacological characteristics. However, there were differences in the magnitude of responsiveness towards agents that were used in this study. The greater contractile responses to mepyramine and angiotensin II in the myofibroblasts which were the predominant cells known to be responsible for contraction in the excisional wound granulation tissues (5) in comparison with fibroblasts which are undifferentiated form of the myofibroblasts, in normal fascia may be due to upregulation of the receptors, increased expression of α-SM isoforms and/or due to presence of greater number of myofibroblastic cells in the granulation tissue relative to normal fascia (2). The underlying mechanism that mediates the contractile responses to relatively high doses of mepyramine, an antagonist to H_1 receptor, is still unknown (10, 20) and can not be explained by findings of the present study. Previous studies also have not explain the underlying mechanisms of responsiveness of granulation tissues from different sources to other antihistamines such as diphenhydramine and promethazine (10, 20, 21).

Previous pharmacological investigations using normal connective tissues did not come to a general consensus and clear conclusions as to the nature of the pharmacological responsiveness of the fibroblasts. While responsiveness of rat testicular capsule to acetylcholine and adrenaline has been demonstrated (19), the responsiveness of the same tissue to diphenhydramine and mepyramine has been reported (20). However, the findings of the former group, could not be repeated by the latter, simply because the former group used the whole of the testicular capsule while the latter group used capsular strips. Furthermore, pathologically induced granulation tissues prepared by various methods did not respond similarly to a variety of pharmacological agents. For example, while croton-oil induced granulation tissue responded to 5-hydroxytrytamine, excisional wound granulation tissue did not (7). On the other hand, $PGF_{2\alpha}$ produced contraction in both human wound granulation tissue (21) and granulation tissues were formed around blood clot (10). However, the former contracted by histamine, while the latter responded to diphenhyramine, an antihistaminic drug. These discrepancies in the reported results suggest that the fibroblasts have a variety of phenotypic forms, four of which, so far, have been identified (2).

The relaxation responses to excess potassium ion, in both tissues employed in the present study contrast to other reports in which these ions produced contraction in smooth muscle cells. One possible explanation is the low resting membrane potential (RMP) of fibroblasts, which was found to be –10.2 mV (22). In the present study the RMP was not measured, and if this assumption is correct, then it is possible that values of RMP of these two cells are expected to be different. This aspect of work deserves further investigation. On the other hand, responsiveness towards excess calcium ion in wound granulation tissues were reduced in comparison to the normal fascia. These findings suggest that during differentiation of fibroblasts to the active contractile myofibroblasts (3), the myofibro-blasts lose their responsiveness, by an unknown mechanism, to excess external calcium and potassium ions. Since excessive calcium ion has adverse effect on the contractility of wound healing (23), it seems that this modification may play a protective role during wound healing process. Differences in the magnitude and duration of responses of wound granulation tissue with those for fascia to agents employed in this study, demonstrates that the myofibroblasts develop into different responsive cells and behave differently from the fibroblasts that they were originated. These observations come in agreement with earlier histological observations in which myo-fibroblasts differentiate morphologically into cells that possess intracellular components that resemble contractile smooth muscle cells (2).

The overall conclusion that may be drawn from this study is that the connective tissue taken from subcutaneous fascia and that prepared from the wound granulation tissues are pharmacologically responsive. However, they have their own distinct pharmacological characteristics. Furthermore, this study showed that during the process of wound healing, the granulation tissue develops changes in its pharmacological responsiveness to agents known to produce effects on this tissue. These changes are of importance if implementation of appropriate wound management protocols is required. Clearly these studies are not conclusive and more intensive studies are required in order to elucidate the underlying nature and mechanisms associated with their pharmacological responsiveness.

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