

Wound Contraction in Relation to Collagen Formation in Scorbutic Guinea-pigs

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INTRODUCTION

A WOUND in any mobile part of the skin of a mammal diminishes in area as it heals by a centripetal movement of the undamaged skin surrounding it. This movement, usually called wound contraction, depends on a pull exerted by the material within the wound (Lindquist, 1946; Abercrombie, Flint, & James, 1954; Billingham & Medawar, 1955). It is commonly believed that the effective force is developed by the newly formed collagen fibres. In a previous paper, however (Abercrombie, Flint, & James, 1954), we found that the course of the contraction of skin wounds in rats did not parallel the deposition of new collagen, chemically measured. This result, while certainly in no way conclusive by itself, suggested that the supposed role of collagen in contraction ought to be tested more stringently. This we have now done by measuring wounds made on guinea-pigs receiving a diet devoid of ascorbic acid. Such a deficiency largely prevents the formation of new collagen (reviewed by Wolbach & Bessey, 1942). We found, however, that it did not prevent wound contraction, a result difficult to reconcile with the usual hypothesis. We have accordingly put forward a new hypothesis as to the causal agent of contraction, which we suggest is a force produced by the population of connective tissue cells within the wound.

MATERIAL AND METHOD

In the main experiment thirty adult male guinea-pigs of mixed stock were used; for convenience of tattooing white or mainly white animals were selected. Their mean body-weight at the beginning of the experiment was 442 g. (range 378–570, standard deviation 43·4). They were tattooed under ether anaesthesia (Abercrombie, Flint, & James, 1954), eight needles being operated simultaneously to mark a square of an area, measured through the centres of the eight tattoo points, of a mean size of 25·5 mm.² (range 18·1–34·4, standard deviation

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4·11). The marks were made in the dorsilumbar region, one square on each side of the mid-line. After tattooing the animals were put on the ascorbic acid deficient diet of Murray & Kodicek (1949) with the salt mixture of Hubbell, Mendel, & Wakeman (1937), but half of them (chosen by random numbers) received in addition a supplement of crystalline ascorbic acid mixed, in the proportion of 1 g. to 100 g., into their food. After 5 days on the diet aseptic operation was performed on each animal under ether anaesthesia. A tracing of the tattoo marks was first taken on tracing paper, and a square wound of about 17 mm.² was made within the tattoo points of one of the pair of squares marked on each animal, the second square remaining for estimation of changes in normal skin during the experiment. The wound extended through the panniculus carnosus to the deep fascia. No dressing was applied. Ten days later the animals were killed. The position of the tattoo marks was again marked on tracing paper, the scab removed (it was necessary to remove the larger scabs before tracing), and the new-formed tissue within the tattoo marks excised. A few animals were rejected on suspicion of infection and we were finally left with material from 14 deficient and 16 non-deficient animals.

The size of each tattoo area, marked by the centres of the tattoo points as described in our previous paper, was obtained by pricking through the tracings on to black paper, cutting out the area enclosed and measuring the transmission of light through the resulting hole photoelectrically, comparing standards of known area. The collagen content of the scabs and that of the excised tissue were obtained separately by the method of Neuman & Logan (1950), which involves estimating hydroxyproline. The results are recorded as milligrammes of hydroxyproline. They may be converted to milligrammes of collagen by multiplying by 7·46.

Mean body-weight on the first day of the diet was 429 ± 9 g. in the deficient group, 453 ± 12 g. in the non-deficient group. The difference is not significant ($t = 1\cdot50$, 28 degrees of freedom, $0\cdot2 > P > 0\cdot1$). Weight-loss during the 15 days of the experiment was practically the same in the two groups (the basal diet was not well taken): in the deficient group it was 46 ± 7 g., in the non-deficient group 45 ± 7 g. The initial tattoo area within which the wound was made measured $25\cdot3 \pm 1\cdot1$ mm.² in the deficient, $25\cdot6 \pm 1\cdot0$ mm.² in the non-deficient. In a sample of 10 of the deficient group and 8 of the non-deficient group the wet weight and hydroxyproline content of the piece excised at the initial wounding were obtained, to check that similar wounds had been made. The mean wet weights of these pieces were a little different though not significantly so (deficients $25\cdot4 \pm 1\cdot8$ mg., non-deficients $21\cdot3 \pm 1\cdot3$ mg.; $t = 1\cdot76$, 16 d.f., $P = 0\cdot1$). The excised tissue of the deficient had a mean hydroxyproline content of 633 ± 45 μ g., that of the non-deficient 577 ± 33 μ g., again a non-significant difference ($t = 0\cdot96$, 16 d.f., $0\cdot4 > P > 0\cdot3$).

RESULTS

Hydroxyproline of repair tissue

At autopsy, after careful removal of the scab, the tissue within the border of the wound marked by the tattoo was excised. It was markedly different in appearance and consistency in the deficient and non-deficient groups. In the deficient group it was very friable, and in the non-deficient control group it was quite firm. Its wet weight was similar in the two groups, with a mean of 13.0 ± 1.7 mg. in the deficient and 10.3 ± 1.3 mg. in the control group. Its hydroxyproline content was, on the contrary, highly dissimilar, with a mean of 10.4 ± 2.6 μ g. in the deficient and 74 ± 8 μ g. in the control group. It is clear that the vitamin deficiency was highly effective in reducing collagen formation. The small amount of hydroxyproline detected in most of the deficient animals (in 3, none was detectable) may, however, represent a trace of reticulin and collagen formed. Silver-impregnated fibres were found in a sample wound fixed in Bouin and stained with Wilder's reticulin method; but no evidence of collagen appeared with Mallory's connective tissue stain. The formation of a small quantity of argyrophilic reticulin (e.g. Bourne, 1944; Penney & Balfour, 1949) and even of mature acidophilic collagen (e.g. Hartzell & Stone, 1942; Danielli, Fell, & Kodicek, 1945) has been described in sections of wounds from scorbutic animals. Wolbach (1933) considered that such fibres do not develop in a totally vitamin C depleted guinea-pig. Since our animals were on the deficient diet for only 5 days before operation it is possible that their reserves of ascorbic acid were not entirely exhausted before healing. There is, however, the possibility to be considered that the hydroxyproline found was not present in the form of collagen but as some soluble precursor or break-down product. We tried but failed to obtain evidence in support of this possibility. In half the specimens the estimation was made of total hydroxyproline regardless of its solubility, and in half the estimation was made after extraction in 20 per cent. urea so that it represented that part of the hydroxyproline having the solubility of collagen. In the deficient group the total hydroxyproline was 13 ± 6 ($N=8$), the collagen hydroxyproline 7.3 ± 2.4 ($N=6$). In the control group the total was 71 ± 11 ($N=7$), the collagen 76 ± 12 ($N=9$). The differences within each group between total and collagen are obviously not significant. For this reason the separate types of estimation have been combined.

Scabs

There was usually a striking difference between the deficient and control animals in the scab covering the wound 10 days after operation. It was usually small both in area and thickness in the controls, averaging 3.7 ± 1.4 mg. wet weight ($N=16$), including three where it had disappeared entirely. In the deficient it was usually very much thicker and larger in area. In this group it averaged 28 ± 6 mg. wet weight ($N=14$), including two where it had disappeared entirely. Some of the scabs of control animals contained traces of hydroxyproline,

as found also at 10 days after wounding in rats (James, 1955): the average for those scabs still present was $10 \pm 8 \mu\text{g}$. ($N = 13$). Most of the scabs from the scorbutic animals, on the other hand, contained substantial amounts of hydroxyproline: their mean was $112 \pm 23 \mu\text{g}$. ($N = 12$). It is unfortunately impossible to determine reliably from these data whether the concentration of scab hydroxyproline was significantly higher in the deficient group or not, since only wet weights are available and water content is not likely to be comparable; and in any case the variances of the two groups are too disparate for proper comparison.

The total hydroxyproline associated with the wound, that is to say both in the scab and in the underlying repair tissue, does not differ significantly between the deficient and control groups. Their means are respectively 121 ± 23 ($N = 14$), and 82 ± 10 ($N = 16$), and comparing these $t = 1.65$, $0.2 > P > 0.1$. This might raise the suspicion that in the deficient animals the hydroxyproline-containing part of the repair tissue was torn away with the large and adherent scab, which would make spurious the difference in hydroxyproline content found between the repair tissue of the deficient and controls. Some damage to the repair tissue did in fact occasionally appear to occur in this way; but the inclusion of an important amount of the hydroxyproline content of the wound with that of the scab should produce a strong negative correlation between scab and wound hydroxyproline, of which there was no trace: the correlation coefficient was positive but non-significant ($+0.10$).

Contraction

Our previous investigation (1954) of skin wounds in rats showed that the difference between the area marked out by the centres of the tattoo points at operation and the equivalent area at autopsy gives a close approximation to (about a 20 per cent. exaggeration of) the contraction of the actual wound within the tattoo points. Measured through the tattoo points, mean contraction in the deficient group during the 10 days of healing was $8.3 \pm 1.0 \text{ mm.}^2$ ($N = 14$); in the non-deficient group it was $10.1 \pm 1.4 \text{ mm.}^2$ ($N = 16$). Significant contraction obviously occurs in the vitamin C deficient group ($t = 8.29$, $P < 0.001$); and the amount that occurs is not significantly different from that of the non-deficient control group ($t = 1.02$, $0.4 > P > 0.3$). In three of the deficient group with *no* detectable hydroxyproline, contraction was 6.8, 9.4, and 12.5 mm.^2 Qualitatively, the contraction appeared to be of the same nature, in that the tension was released when the content of the wound was cut free from the margin of normal skin. Our data therefore fail to provide any evidence that contraction is dependent on collagen formation. Nevertheless, the small difference actually found was in favour of the control group; and when both groups are pooled, the six wounds with most contraction all belong to the non-deficient group. It might be suspected that with larger samples the slight difference would become significant. This may be so, but the data do not suggest that such a difference could then be connected with the hydroxyproline content of the repair tissue. In fact in both deficient and

control groups wound hydroxyproline and contraction were *negatively*, though not significantly, correlated ($r = -0.26$ in both groups). The scab might have been expected to obstruct contraction in the deficient group; and scab weight was indeed negatively correlated with contraction in this group ($r = -0.35$) but again the relation is not significant at the 5 per cent. level of probability.

Changes in normal skin

There was a small and non-significant diminution of the area of the tattoo on the control side during the 15 days between the start of the diet and autopsy in a sample of both deficient and non-deficient groups (diminution of 0.7 ± 2.2 mm.², $N = 8$; and 0.8 ± 0.7 mm.², $N = 6$ respectively). The possibility that the deficient diet might produce detectable loss of collagen was also investigated in a small sample. No significant loss of collagen could, however, be detected when we compared the content of the piece removed at operation with the content of the control area which was removed at autopsy. At operation the mean content was 608 ± 33 μ g. ($N = 9$), at autopsy 700 ± 70 μ g. ($N = 9$). At autopsy the weight of the piece removed was rather higher than at initial operation, though not significantly so; probably this was the result of a slight bias in choosing the best-marked side for operation. Use of analysis of covariance to eliminate the size difference left the hydroxyproline difference still quite non-significant ($t = 0.383$, $P = 0.7$).

DISCUSSION

The inhibition of collagen formation in healing wounds by ascorbic acid deficiency has been demonstrated many times before (see Wolbach & Bessey, 1942), though apparently not previously by a chemical method. The absence of any effect of the deficiency on the amount of already formed collagen has been shown chemically (Elster, 1950; Robertson, 1950, 1952). The conspicuous and persistent scabs that form over wounds of scorbutic guinea-pigs were noted by Wolbach & Howe (1926), Hartzell & Stone (1942), and Danielli, Fell, & Kodicek (1945). It is not clear why they should be so different from the scabs of normal animals, but the increased fragility of the vessels (Lee & Lee, 1947) may mean a greater production of exudate; and delayed epithelialization (Danielli, Fell, & Kodicek, 1945; Galloway, Garry, & Hitchin, 1948) may allow this to accumulate. We have, however, no reason to believe that epithelialization was delayed in our specimens: the presence of the scab does not necessarily mean a failure of healing, since one specimen with a particularly large scab, which was examined histologically, had a complete and hyperkeratinized epidermis adherent to the underside of the scab. Because of the absence of collagen, such epidermis is easily torn away when the scab is removed, and the wound then looks unhealed. The presence of hydroxyproline in the scabs of wounds in rat-skin has already been demonstrated by James (1955). It remains uncertain whether scab hydroxyproline represents a diffusible potential precursor of new collagen, or comes from degeneration of some of the original collagen bordering the wound. Dévényi &

Holzinger (1954) have described the incorporation of degenerating connective tissue of the wound floor into the scab. It is not at present worth speculating on the difference in scab hydroxyproline between control and scorbutic animals. It may merely be a reflection of the curious persistence of the scab in the scorbutic animals, and not of any difference in hydroxyproline production.

The main purpose of this work was to investigate the relation between wound contraction and collagen formation. We found that wound contraction was not significantly reduced by the very severe inhibition of collagen formation that resulted from the ascorbic acid deficiency. On the average only 15 per cent. of the amount of hydroxyproline in the wounds of the controls was present in the wounds of the scorbutics, and three of the latter contracted even though devoid of detectable hydroxyproline. The contraction in both groups involved a diminution of the actual wound area by probably about 30–40 per cent., a considerable proportion, though rather less than occurs during the first 10 days in the healing of a similar-sized wound in the rat, where it is about 60 per cent. (Abercrombie, Flint, & James, 1954). The traditional hypothesis implicating newly formed collagen will not reasonably account for this contraction. Nor does there seem to be any good reason for suggesting that some intercellular component other than collagen is responsible. An intercellular substance is formed in scorbutic wounds. It has been described by Wolbach (1933), Penney & Balfour (1949), and Bradfield & Kodicek (1951). It is, however, histologically and histochemically quite different from that of normal wounds. It is appropriate therefore to suggest a new hypothesis, bearing in mind that perhaps the whole range of phenomena included under wound contraction in man and other species may require more than one explanation. The new hypothesis is that the contractile force is produced by the connective tissue cells that occupy the wound. Many authors have remarked that cellular proliferation in wounds is undiminished, or even slightly increased, by ascorbic acid deficiency (Wolbach, 1933; Hunt, 1941; Hartzell & Stone, 1942; Bourne, 1944; Meyer & Meyer, 1944). The fibroblasts may not be normal in form (Penney & Balfour, 1949) or in arrangement (Danielli, Fell, & Kodicek, 1945; Meyer & Meyer, 1944), and their immigration may be delayed (Mazoué, 1937). But, at least as far as microscopic observation extends, they are much less affected than is the intercellular substance. This is in contrast with wounds in animals treated with cortisone. Here both collagen formation and the recruitment of new cells is depressed. It is therefore not surprising, from the standpoint of the hypothesis we are putting forward, that contraction is inhibited too (Billingham, Krohn, & Medawar, 1951 *a, b*).

In one respect cells are a more obvious first choice for a contractile mechanism than is collagen, since neither a reticulin meshwork nor individual collagen fibres have ever been shown to be contractile under physiological conditions, but contractile mechanisms obviously occur at least in some cells. Smooth muscle-cells in tissue culture are indistinguishable from ordinary connective tissue fibroblasts; and it is conceivable that the contractile power of muscle-cells is

widespread in a primitive form throughout the fibroblast family (see Hoffmann-Berling, 1954). This line of thought, however, perhaps directs attention too much to a contractile mechanism intrinsic to the cell. Another possibility is that the force may be produced by the mutual rearrangement of cells, perhaps by expansion of the areas of adhesion which exist between cells in a fibroblast colony (Kredel, 1927), so packing them more closely together. Whatever the mechanism, there is no doubt that cells that are not muscle-cells can exert a tractive force. Mayer (1933) has shown that considerable tensions exist in fibroblast cultures which can be ascribed to the cells. Twitty (1949) has found that melanoblasts of *Triturus* under certain circumstances draw themselves together, and this is important in the formation of pigment patterns. Finally, a possibly analogous phenomenon is the contraction of blood clots, which Budtz-Olsen (1951) has shown is due not to the fibrin but to the platelets.

If it can be demonstrated that a population of fibroblasts can develop forces of the magnitude of those required to cause wound contraction, the phenomenon may have a wider significance. The embryo might in this way be provided with a source of motive power which could be a cause of the torsions, flexures, and transport of entire organs which occur in development.

SUMMARY

1. In order to investigate the relation of wound contraction to collagen formation, standard skin wounds were made on two groups of guinea-pigs, one group kept on an ascorbic acid deficient diet, the other on the same diet supplemented with ascorbic acid.

2. Ten days after wounding the amount of collagen in the repair tissue, estimated by the method of Neuman & Logan (1950), was much less in the deficient than in the non-deficient animals.

3. Nevertheless, the amount of wound contraction which had occurred, representing a loss of 30–40 per cent. of the original wound area, was not significantly different in the two groups.

4. Large amounts of hydroxyproline were found in the massive scabs commonly developed over the wounds of scorbutic animals.

5. It is suggested that the force that brings about wound contraction may be developed by the connective tissue cells of the repair tissue.

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