577 . 16(C) : 616—001 . 4—003 . 9

THE EFFECT OF VITAMIN C ON MUCOPOLYSACCHARIDE PRODUCTION IN WOUND HEALING

J. R. PENNEY and BRIGID M. BALFOUR

Human Nutrition Research Unit, Medical Research Council

(PLATES XXXV-XXXVII)

HISTOLOGICAL studies of experimental wounds from vitamin C-deficient guinea-pigs have led to the conclusion that impaired healing is associated with failure to form extracellular material (Wolbach, 1933; Hunt, 1940-41). Similar results have been obtained in human experiments (Crandon, *et al.*, 1940; Hunt, 1940-41; Medical Research Council, Accessory Food Factors Committee, 1948).

Wolbach has described in some detail the appearance of sections of wounds from depleted guinea-pigs. He found that, although proliferation and migration of fibroblasts occurred, no extracellular material was formed. However, soon after the administration of vitamin C to the depleted animals a homogeneous amorphous material which stained blue with Mallory's connective tissue stain appeared round the cells. This was followed rapidly by the formation of reticulin fibres embedded in the amorphous material. The production of this material is of interest, since it is the first indication that the repair process has been resumed. The chemical nature of this amorphous material was not established by Wolbach who, for convenience, called it amorphous collagen. Sylvén (1941), studying the formation of granulation tissue in wounds from normal animals, found that substances which stained metachromatically with toluidine blue appeared very early in the repair process. The dye was considered to be specific for mucopolysaccharide sulphuric acid esters. The presence of mucopolysaccharides may be inferred from results of Bensley (1934) on the properties of the material extractable from granulation tissue. In chronic scurvy, Meyer (1943-44) found that the metachromasia of articular cartilage with toluidine blue was greatly reduced, due, he considered, to reduction of the chondroitin sulphate content. The "homogeneous collagen" of Wolbach may, therefore, be "mucopolysaccharide," the formation of which may be dependent on the supply of ascorbic acid.

In this communication we report observations on the chemical nature of the extracellular material produced in the initial stages of normal wound healing and give the results of experiments on the relationship between vitamin C and the formation of this material.

Methods

Growing guinea-pigs of approximately 300 g. weight were maintained on a basal vitamin C-free diet (Penney and Zilva, 1946). For the study of normal

repair, the animals were given either 25 mg. of ascorbic acid daily or cabbage *ad lib.* in addition to the basal diet. Muscle wounds were made as described by Wolbach; after given intervals the animals were killed by stunning and bleeding and the wound area taken for histological study. Twenty-three normal guinea-pigs were killed at daily intervals from the 4th to the 9th day after operation. The remaining animals were wounded after 13 days on the vitamin C-free diet and were either killed at daily intervals from the 5th to the 9th day after operation (17 animals), or injected intramuscularly with 25 mg. of ascorbic acid on the 7th day after wounding and killed 6, 12, 18, 24 or 48 hours later (11 animals). Immediately after death the wound area was excised, fixed in 10 per cent. neutral formalin for 24 hours, in Helly's fluid or in 80 per cent. alcohol and embedded in paraffin.

For general histological studies the stains used were hæmatoxylin and van Gieson, Foot's modification of Bielschowsky's silver impregnation method, Weigert's iron hæmatoxylin and Unna's methyl-green pyronin.

For the demonstration of acid mucopolysaccharides, the metachromatic staining properties of toluidine blue were utilised, following, usually, the procedure described by Sylvén. We have found that there is a considerable variation between different batches of the dye. Some samples gave satisfactory metachromatic staining after ordinary alcohol dehydration. With other samples the metachromasia, although visible when examined under water, was destroyed by alcohol, but in some cases could be preserved by using dioxane as the dehydrating agent. This variation in the behaviour of the stain may account for the different staining techniques reported in the literature. In this investigation every batch of dye was tested on control sections taken from the same block and only those which gave good metachromatic staining after alcohol or dioxane dehydration were used.

Although the metachromasia with toluidine blue was originally believed to be specific for mucopolysaccharide sulphuric acid esters (Lison, 1936), it was later reported that this effect is also produced by nucleoproteins (Wislocki *et al.*, 1947) and hyaluronic acid in high concentration (Meyer, 1947). We therefore attempted to increase the specificity of the method by observing the effect of hyaluronidase and ribonuclease on the substances which exhibit metachromasia. In the first instance de-paraffinised sections were treated with purified ox-testis hyaluronidase in one per cent. saline (activity 2000 M.C.U./ml.) or with one per cent. saline alone for four hours at room temperature (20°-22° C.). Sections from the same blocks were incubated with purified ribonuclease (10 mg./100 ml.) at *p*H 6·7 or with buffer solution alone for 1 hour at 37° C. After treatment, all the sections were stained with toluidine blue and compared with the untreated sections.

Results

Normal group. In this group fibroblastic and vascular invasion of the clot was observed 4 days after wounding. By the 5th day several stages of the repair process could be seen in sections from the same wound. At the periphery of the wound, elongated and orientated fibroblasts were found associated with van Gieson-staining fibres. Fibroblasts which had advanced further into the clot were triangular or rhomboidal and were usually in the form of a syncytium. Stearns (1940) has reported that this arrangement precedes fibre formation. Associated with the fibroblasts were both fine and coarse reticulin fibres. After staining with toluidine blue, metachromasia was evident in the cytoplasm of the fibroblasts and in the extracellular material.

A close correlation was noted between the distribution of newly formed fibres and the extracellular metachromasia. In areas where the fibroblasts had migrated deeply into the clot, the metachromatic staining material was sometimes found in amorphous form with very fine argyrophil fibres embedded in it. Usually, however, it had a fibrous structure, resembling the pattern of both the fine and coarser reticulin fibres (figs. 1 and 2), suggesting that the argyrophil fibres themselves possessed metachromatic staining properties. Towards the periphery of the wound area, the coarser bundles of toned and van Gieson-staining fibres were clearly metachromatic. On increasing the post-operative period to 9 days, more van Gieson-staining fibres were observed. As these fibres mature, they lose their metachromatic staining properties (*cf.* Sylvén).

In addition to the metachromasia associated with fibroblasts, areas of amorphous material which gave the characteristic metachromatic staining could be found far removed from the cells in the depth of the wound.

Depleted group. When sections of wounds from this group taken five days after operation were examined, it was found that some fibroblastic proliferation had occurred. The cells had migrated into the clot, but as noted by Wolbach they were very abnormal in form and distribution. They were pleomorphic and rarely attained the characteristic appearance of normal fibroblasts. Many cells had small densely staining nuclei and scanty amounts of cytoplasm, while in others a hyperchromatic nuclear membrane surrounded an almost clear nucleus with no nucleolus. Cells with large vacuoles, generally found near those engaged in phagocytosis, are probably histiocytes; in some areas these were the predominating cell types. Very small vacuoles were sometimes observed in the abnormal fibroblasts; these appeared to correspond to small fat droplets found in frozen sections.

The distribution of the cells in the wound area differed greatly from that found in normal wounds. The fibroblasts were generally separated and never formed a syncytium except in small areas near the periphery of the wound. In these areas the cell form closely resembled that of a normal fibroblast.

Although the cells had proliferated and migrated into the clot, very little extracellular material had been formed (fig. 3). Since, on increasing the post-operative period up to 9 days, no further attempts at repair appeared to be made, the following remarks refer to sections taken over the whole period studied.

Small areas of reticulin fibres associated with metachromasia were sometimes observed at the periphery of the wound, where the fibroblasts appeared less abnormal. It is of interest that occasionally these areas were in close proximity to the frayed and disintegrating ends of pre-formed collagen fibres. In this condition the collagen exhibited metachromasia and became argyrophil. As the fibroblasts penetrated further into the clot, however, no attempt at fibre formation

was observed and no metachromatic staining material associated with cells could be found, either with the formol- or Helly-fixed material. Amorphous material which stained metachromatically was sometimes observed in the clot away from fibroblasts, resembling, in this respect, the normal wounds.

Metachromasia of the cytoplasm was not a constant finding. Those cells which were almost normal in appearance had this property, but the abnormal cells with scanty cytoplasm varied in their staining reactions.

Limited capillary proliferation was also a characteristic of depleted wounds and the new-formed capillaries were often abnormal. They frequently failed to form a lumen and corresponded to the closed columns of endothelial cells described by Wolbach (fig. 4). In addition, heaping up of the cells around patent vessels could be observed (fig. 5). Differentiation of the vessels, obvious in the normal process of repair, did not take place. It is clear from these observations that the blood supply to the more remote parts of the wound area is inadequate and the abnormal appearance of some of the cells in these areas may be attributable to inanition.

After the intramuscular administration of ascorbic acid to depleted animals on the 7th day of the post-operative period (20th day of complete depletion), striking differences in staining properties and cell morphology were observed. A large increase in the amount of metachromatic staining material was found after 12 hours, and even after 6 hours there was evidence of increased production of these substances. By 12 hours it was noted that some isolated cells which had penetrated into the clot were surrounded by confined regions of amorphous material showing metachromasia. Very fine reticulin fibres were distributed through some but not all of these areas. In regions containing considerable numbers of cells much extracellular material had been produced and fine reticulin fibres associated with metachromasia were much in evidence (figs. 6 and 7). On increasing the interval after ascorbic acid administration, fibre formation took place rapidly and by 24 hours van Gieson-staining and toned fibres had appeared.

Vigorous cellular activity was also noted in the perivascular connective tissue surrounding the larger arteries. Here it appeared that the cells were concerned with newly formed fibrous extracellular material which stained metachromatically but was not argyrophil.

There was a rapid change in the morphology of the cells accompanying the formation of extracellular material. In 12 hours many of the cells had attained the appearance of normal fibroblasts. Both cytoplasm and nuclei had enlarged and the latter had become vesicular, with prominent nucleoli. After this interval the cytoplasm of the normal and of some of the abnormal cells was metachromatic. It should also be noted that some of the cells which had not yet

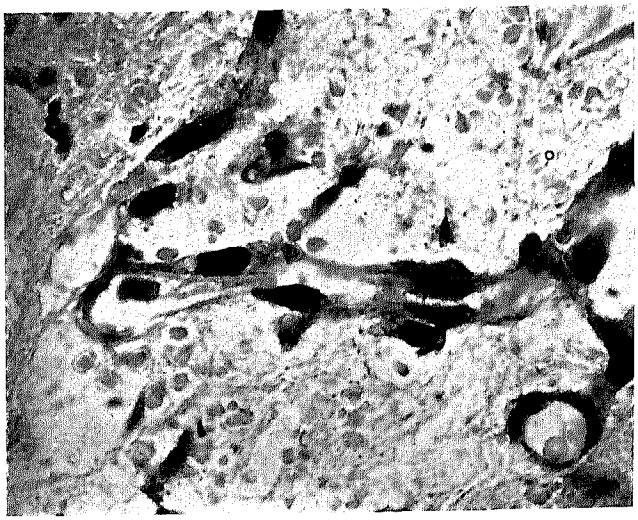

FIG. 8.—Vascular proliferation following ascorbic acid administration to initially vitamin C-depleted animal. Formation of lumen in non-patent vessel. Weigert's iron hæmatoxylin. ×700.

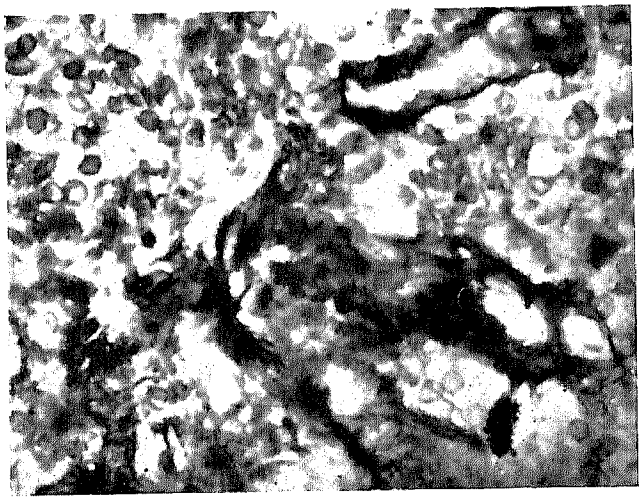

FIG. 9.—Vascular proliferation following ascorbic acid administration to initially vitamin C-depleted animal, showing perivascular reticulin sheath appearing 18 hours after ascorbic acid administration. Foot's modification of Bielschowsky's silver stain. ×783.

attained the appearance of normal fibroblasts were associated with newly formed extracellular material. Forty-eight hours after ascorbic acid administration all the cells were normal in appearance.

The development of capillaries also responded rapidly to ascorbic acid therapy. During the experimental period, non-patent vessels developed a lumen ; this change was more noticeable at the periphery of the wound (fig. 8). In areas containing newly formed reticulin fibres it was sometimes found that fine fibres had become attached to the vessel wall. Where a vessel had penetrated into the clot unaccompanied by fibroblasts, argyrophil fibres formed a definite perivascular sheath (fig. 9).

Enzyme treatment of sections

In these experiments, sections were taken of 7-day wounds from control and depleted animals and from injected animals. After treatment with hyaluronidase, or 1 per cent. saline, the sections were stained with toluidine blue and compared with untreated sections. One per cent. saline did not affect the resulting metachromasia. Hyaluronidase, however, removed the substances responsible for the metachromasia in the newly formed extracellular material (figs. 10 and 11) leaving a non-metachromatic residue. This provides good evidence that these substances are mucopolysaccharides of the hyaluronic acid or chondroitin sulphate type. The metachromatic material of the cytoplasm did not appear to be affected by hyaluronidase.

We should like to record that the metachromatic staining of disintegrating collagen, of amorphous material in the depths of the wounds, of the media of larger blood vessels and of the perineurium was abolished by hyaluronidase, whereas the granules of mast cells were unaffected.

After treatment with ribonuclease no metachromasia could be observed in the cytoplasm, but the extracellular material was unaffected. Selective removal of the metachromasia from the cytoplasm of the endothelial cells sometimes revealed very fine metachromatic staining fibres outlining capillaries. This could be seen more easily in the wounds taken from the injected animals 12 hours after ascorbic acid administration.

Discussion

The appearance of substances which stain metachromatically in granulation tissue, described in some detail by Sylvén, has been confirmed. On the basis of this staining property he suggested that both the cytoplasmic and extracellular metachromasia were due to the presence of mucopolysaccharide sulphuric acid esters. Our observations using toluidine blue in conjunction with testicular hyaluronidase indicate that the extracellular substance or substances giving rise to metachromasia are acid mucopolysaccharides but we do not feel justified in attempting to characterise these substances

more definitely at this stage. The possibility of hyaluronic acid giving metachromasia cannot be excluded (Stacey, 1946; Meyer, 1947; Wislocki *et al.*, 1947), although it should be remembered that we took no special precautions to retain it (*cf.* Leach, 1947). Furthermore, the enzyme used by us is known to degrade both hyaluronic acid and chondroitin sulphate (Humphrey, 1946). The use of the specific streptococcus hyaluronidase might help to elucidate this point.

The metachromasia of the fibroblasts is probably due to the presence of ribo-nucleoproteins, since it can be removed by ribonuclease. This is in keeping with the high cytoplasmic nucleic acid content found in growing and protein-secreting cells.

The failure of wound repair in depleted animals is associated with the failure of formation of extracellular material in both homogeneous and fibrous form. It is of interest that the stage at which the repair process is retarded depends on whether the supply of ascorbic acid is completely withheld or whether sub-minimal amounts are supplied. Hence Danielli *et al.* (1945) found that on low doses of ascorbic acid (less than 2 mg. per day) large amounts of reticulin could be formed although the wound appearance was not normal. In preliminary experiments we have obtained indications that on these low doses of ascorbic acid large amounts of mucopolysaccharide are formed.

The appearance of mucopolysaccharide and reticulin after the injection of ascorbic acid to depleted animals was very rapid, and was accompanied by a change in cell morphology. Since mucopolysaccharide was found associated with fibroblasts which were still abnormal, we are inclined to the view that resumption of the reparative process precedes the attainment of normal cell morphology. Within 12 hours of ascorbic acid administration considerable amounts of mucopolysaccharide were found, and although areas of homogeneous material were sometimes found alone, the mucopolysaccharide was very often associated with fine reticulin fibres. Wolbach was able to demonstrate more clearly the production of a homogeneous extracellular material using aniline blue, and by virtue of its staining properties termed the material " amorphous collagen." The distribution of this material appears to correspond very closely to that of the mucopolysaccharides found in our experiments. It is probable that by the injection of large doses of ascorbic acid the response in our experiments was more rapid and the successive stages not so clearly defined as in Wolbach's experiments, in which the animals were given orange juice. Similarly the difficulty in observing well-defined stages in normal healing is probably due to the rapidity of the process.

From the evidence available it would appear that the first event leading to fibre formation is the deposition of mucopolysaccharide about the fibroblasts in homogeneous form. These substances are known to occur in nature in association with protein and we have observed a non-metachromatic-staining residue in sections after

treatment with hyaluronidase. This residue might be responsible for the staining results obtained by Wolbach with aniline blue. We do not, however, consider that the available evidence is sufficient to indicate that a true homogeneous protein precursor of collagen is present, as suggested by Meyer (1947). The initial production of mucopolysaccharide is followed by the formation of very fine argyrophil fibres embedded in the homogeneous material. Thickening of these fibres is accompanied by a change in the distribution of the mucopolysaccharide, which now takes a fibrous form corresponding to the argyrophil fibres. This suggests that the fibre protein is closely connected with the mucopolysaccharide, either in physical combination (Meyer, 1947) or as a complex. After maturation of the fibres, the mucopolysaccharide can no longer be demonstrated histologically. The mechanism of fibre formation is not yet understood, although the in-vitro production of fibres can be brought about by acidification of a solution containing gelatin and chondroitin sulphate (Meyer *et al.*, 1937). Whether a similar mechanism is involved in the in-vivo production of fibres is obscure.

The possibility of mucopolysaccharide disturbance in scurvy has been reported by Meyer (1943-44), who found that the metachromasia of articular cartilage was very much reduced in the chronic form of this disease. Such a disturbance might also account for the hæmorrhages associated with the deficiency. Recently Chambers and Zweifach (1947) have obtained evidence for the presence of mucopolysaccharide in the connective tissue sheath of the blood capillaries. Any failure in mucopolysaccharide formation might result in the weakening of the sheath, leading to hæmorrhages. It is of interest that the connective tissue sheath associated with proliferating vessels can be demonstrated histologically by its metachromatic properties.

Summary

1. The production of acid mucopolysaccharides in the early stages of normal wound healing in guinea-pigs has been confirmed.

2. There is a failure in the production of these substances in the wounds of guinea-pigs depleted of vitamin C.

3. The intramuscular injection of ascorbic acid to depleted animals results in the prompt appearance (within twelve hours) of mucopolysaccharides in the wound.

4. The sequence of events in wound healing is discussed.

We wish to express our thanks to Professor B. S. Platt for his interest and criticism throughout this work, to Professor J. M. Davidson and Dr J. H. Humphrey for gifts of purified ribonuclease and hyaluronidase, to Messrs Roche Products Ltd. for a gift of ascorbic acid, and to Miss D. P. Woodnott and Mr R. F. Preece for technical help and the production of the photomicrographs.

REFERENCES

Bensley, Sylvia H.	1934.	*Anat. Rec.*, lx, 93.
Chambers, R., and Zweifach, B. W.	1947.	*Physiol. Rev.*, xxvii, 436.
Crandon, J. H., Lund, C. C., and Dill, D. B.	1940.	*New Engl. J. Med.*, ccxxiii, 353.
Danielli, J. F., Fell, Honor B., and Kodicek, E.	1945.	*Brit. J. Exp. Path.*, xxvi, 367.
Humphrey, J. H.	1946.	*Biochem. J.*, xl, 442.
Hunt, A. H.	1940-41.	*Brit. J. Surg.*, xxviii, 436.
Leach, E. H.	1947.	*Stain Technol.*, xxii, 73.
Lison, L.	1936.	Histochimie animale, *Paris*, pp. 236-242.
Medical Research Council .	1948.	A preliminary report by the Vitamin C Subcommittee of the Accessory Food Factors Committee. *Lancet*, i, 853.
Meyer, A.	1943-44.	*Z. Vitaminforsch.*, xiv, 332.
Meyer, K.	1947.	*Physiol. Rev.*, xxvii, 335.
Meyer, K., Palmer, J. W., and Smyth, Elizabeth M.	1937.	*J. Biol. Chem.*, cxix, 501.
Penney, J. R., and Zilva, S. S. .	1946.	*Biochem. J.*, xl, 695.
Stacey, M.	1946.	Advances in carbohydrate chemistry, *New York*, ii, 161.
Stearns, Mary L.	1940.	*Amer. J. Anat.*, lxvii, 55.
Sylvén, B.	1941.	*Acta chir. Scand.*, lxxxvi, suppl. 66.
Wislocki, G. B., Bunting, H., and Dempsey, E. W.	1947.	*Amer. J. Anat.*, lxxxi, 1.
Wolbach, S. B.	1933.	*Amer. J. Path.*, ix, 689.