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AUTORADIOGRAPHIC INVESTIGATION OF THE RATE OF COLLAGEN SYNTHESIS DURING STIMULATION OF WOUND HEALING

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The rate of tropocollagen synthesis by fibroblasts of granulation tissue and of its liberation into the intercellular space was investigated in control animals and during stimulation of the wound process by potassium orotate, a pyrimidine derivative. Under the influence of the stimulator tropocollagen synthesis was observed to be increased. The precursor of collagen fibers appeared in the intercellular space and was incorporated into the fibrous structures of the granulation tissue sooner under these circumstances than in the control, a fact which correlated with the intensification of RNA synthesis in the fibroblast nuclei and with the more rapid liberation of the newly synthesized RNA from the nucleus into the cytoplasma of the cells under similar conditions. No sharp excess of collagen was observed in the granulation tissue of the animals receiving potassium orotate, however.
KEY WORDS: tropocollagen; proline-^{3}H; potassium orotate; fibroblasts; rate of collagen synthesis.

Investigations during the last 10-15 years have resulted in the opinion that collagen is synthesized by fibroblasts and then transported into the intercellular space [1, 2, 4]. So far as wounds are concerned, the periods of maximal synthesis of collagen molecules and formation of collagen fibers have been established [8-11]. It has also been shown that under conditions of hypo- andavitaminosis C the quantity of newly formed collagen in wounds of guinea pigs is sharply reduced but it retuns quickly to normal if the animals are transferred to a diet containing sufficient vitamin C [7, 14, 15]. However, hardly any work has been done to discover the effect of stimulation of the wound process on the rate of formation of collagen and the quantity of it formed de novo. By applying the method of autoradiography using the labeled precursor of collagen such an analysis can be undertaken.

With these facts in mind it was decided to investigate the rate of formation of tropocollagen in fibroblasts and of liberation of the newly formed precursor of collagen fibers into the intercellular space during stimulation of the wound process by potassium orotate, a pyrimidine derivative, and also to try to establish quantitative differences in the content of newly synthesized collagen in the granulation tissue of experimental and control animals.

EXPERIMENTAL METHOD

Experiments were carried out on 30 noninbred albino mice weighing 25 g. In the region of the anterolateral surface of the thigh an incision 1 cm long was made in the skin and subcutaneous cellular tissue and a piece of muscle measuring 2 × 2 × 2 mm was removed. The wounds healed beneath a scab. The animals were divided into two groups. Mice of one group immediately after the operation began to receive potassium orotate by mouth as a 2% solution in a dose of 0.2 ml daily and the animals of the other group acted as the control.

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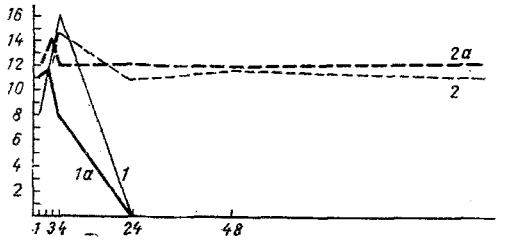

Fig. 1

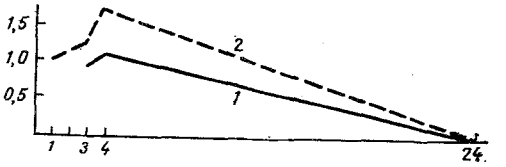

Fig. 2

Fig. 1. Number of grains of silver per unit area in fibroblasts and intercellular space: 1 and 1a) number of grains of silver in fibroblasts in control and experimental group respectively; 2 and 2a) number of grains of silver in intercellular space in control and experiment respectively. Ordinate, number of grains of reduced silver per unit area; abscissa, time after injection of isotope (in h).

Fig. 2. Ratio between number of grains of silver in intercellular space and number in fibroblasts per unit area: 1) control; 2) experiment. Ordinate, ratio between number of grains of silver above intercellular space and number above fibroblasts; abscissa, time after injection of isotope.

For autoradiography proline-^{3}H was injected intraperitoneally into the animals in a dose of 20 μCi/g body weight on the fifth day after wounding. This time was chosen on the grounds that in previous experiments [3] on animals receiving potassium orotate by the same scheme, mature collagen fibers appeared on the wound surface by the fifth day after the operation, coinciding with a period of intensive RNA synthesis in the fibroblasts of these animals. Tissue from the region of the wound was fixed 1, 3, 4, and 24 h and 2 and 7 days after administration of the isotope. The material was fixed in a 10% solution of neutral formalin and embedded in paraffin wax. Parallel with this, to obtain sections 1μ thick, pieces of granulation tissue were fixed in 2.5% glutaraldehyde solution and 1% osmium tetroxide solution and then dehydrated in alcohols of increasing strength and embedded in a mixture of Epon and Araldite. Paraffin sections 4-5 μ thick and semithin sections mounted on slides were coated with M emulsion and exposed at 4°C depending on the thickness of the sections for 2 and 4 weeks. After development, the parathin sections were stained with hematoxylin–eosin and by Van Gieson's and Masson's methods, with some modification of the last of these methods. Semithin sections were stained with 1% methylene blue solution mixed with 1% borax solution.

Grains of reduced silver were counted above fibroblasts and the intercellular space and then their number calculated per unit area of section. The results were subjected to statistical analysis by Wilcoxon's method.

EXPERIMENTAL RESULTS

By the time of injection of the labeled precursor of collagen, i.e., on the fifth day after wounding, granulation tissue was well developed in both groups of animals. However, its layer in the experimental mice was larger. The granulations were well vascularized. The fibroblasts had the typical structure with well developed long processes and large nuclei containing two or three nucleoli. Bands of fibroblasts were arranged parallel to the completely epithelized wound surface. Numerous grains of reduced silver were concentrated above the cytoplasm of the cells. The granulation tissue was less well developed in the control animals. Its layer was thinner. Together with mature forms of fibroblasts, fusiform cells and young fibroblasts, containing no isotope whatever or very small quantities of it, also were present in it. The wound surface was covered with a thin layer of regenerating epidermis.

During the first 3 h after injection of proline-^{3}H the label in the control animals was distributed mainly above the fibroblasts. One hour after injection of the isotope the fibroblasts contained very small amounts of labeled proline, and none whatever could be seen in the intercellular space. Accumulation of the label in the intercellular space did not begin until 3 h after injection of the isotope. After 4 h the intercellular space of the granulation tissue of the control animals already contained large quantities of labeled proline, but as before most of it was concentrated inside the fibroblasts (Fig. 1).

In sections stained by Masson's method the label could be found in the fibrous structures of the granulation tissue. In the experimental animals 1 h after injection of the isotope, the number of grains of silver

calculated per unit area was about equal above the fibroblasts and the intercellular space. The concentration of isotope in the intercellular space 3 h and, in particular, 4 h after injection of the labeled proline was higher than its concentration in the fibroblasts. Differences between the experimental and control series were statistically significant (Fig. 1). Isotope was absent from the fibroblasts in both groups 24 h after injection of the collagen precursor. Label was found only above the intercellular space. This was confirmed by investigation of autoradiographs obtained with sections 1 μ thick.

During stimulation of wound healing not only was the time of synthesis of collagen molecules in the cytoplasm of the fibroblasts shortened, but the newly formed precursor of the collagen fibers appeared in the intercellular space sooner than in the control. This was demonstrated in Fig. 2, where the ratio between the amounts of isotope in the intercellular space and its quantity within the fibroblasts of the experimental and control animals is shown graphically.

Counting the grains of silver above the intercellular space 2 and 7 days after injection of the isotope, i.e., on the 7th and 12th days after the operation, revealed only a very small excess of isotope in the granulation tissue of the animals receiving potassium orotate.

Comparison of these results with those of the investigation of RNA synthesis in the fibroblasts of granulation tissue at the same times of the postoperative period in control and experimental animals [3] showed that under the conditions of stimulation the intensification of RNA synthesis in the fibroblasts and the more rapid transfer of the newly formed RNA from nucleus into cytoplasm correlate with the more intensive synthesis of tropocollagen and increased liberation of the newly formed precursor of collagen fibers into the extracellular space. However, the high level of synthesized RNA in fibroblasts of granulation tissue in the animals receiving potassium orotate was not accompanied by accumulation of a sharp excess of collagen in the intercellular space. This situation can be explained on the assumption that fibroblasts synthesizing tropocollagen and fibroblasts synthesizing enzymes participating in the lysis of excess collagen exist simultaneously in granulation tissue. Under the influence of the stimulator both populations perform their functions more intensively.

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