Changes in the RNA of Cellular Nuclei During Wound Tissue Regeneration[1] (35487)

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The protein content of wound tissue increases rapidly during the first 7–9 days of regeneration; thereafter, although the rate of formation decreases, protein continues to accumulate (1, 2). A better understanding of protein metabolism in this tissue may arise from a knowledge of RNA metabolism.

Extensive breakdown of RNA occurs in granulation tissue shortly after wounding (3, 4). After the initial degradative period, the total cellular RNA per gram of this tissue increases rapidly and reaches a maximal level, also in about 7–9 days. Thereafter, a decrease in RNA is observed (3–6).

Besides qualitative changes, the rate of RNA synthesis increases in the nuclei of regenerating liver as compared to the normal (7–9). Similar information about the metabolic perturbations of RNA in regenerating wound tissue is not available. In this report, we present the results of both qualitative and quantitative studies on nuclear RNA in granulation tissue at different stages of regeneration.

Experimental Methods. Nuclear RNA metabolism during wound tissue formation was studied in virgin female rats, weighing 200 ± 10 g at the start of the experiment. The rats were wounded by excising a circular piece of skin (4 cm in diam) down to the loose fascia from the scapular region while the animals were under Nembutal anesthesia (5). Five days after wounding, the regenerating tissue, from four groups of 20 rats each, was excised and immediately frozen in liquid nitrogen. In separate groups of 20 identically treated rats, the regenerating tissue was harvested 3 hr after the subcutaneous administration of 45 μCi of 5-^{3}H-L-proline to measure the relative rate of protein synthesis. Granulation tissue from similar groups of rats was obtained in the same way after 8 and 12 days of regeneration.

Pooled tissue samples from 20 rats were pulverized in a steel mortar with Dry Ice. They then were homogenized in 0.25 *M* sucrose containing 0.003 *M* Ca^{2+} in 0.05 *M* Tris buffer, pH 7.2, at 2–4° (10). The nuclei, mixed with large amounts of collagen fibers, were centrifuged at 600*g*. Histological examination revealed no intact cells in the precipitate and less than 5% of cytoplasmic fragments. The isolated nuclei were suspended in 10.0 ml of 0.05 *M* Tris buffer (pH 7.2), and ruptured with 1.0 ml of 2% sodium lauryl sulfate. To the lysed nuclei was added 10.0 ml of freshly distilled 88% phenol, and the mixture was shaken for 1 hr at 4°. It then was centrifuged at 25,000*g*. Three more extracts were obtained from the phenolic phase with 10.0 ml portions of the same Tris buffer. Since the fourth fraction contained only about 3% of the total nuclear RNA extracted, it was combined with Fraction 3. The residual phenolic phase then was extracted 3 times with 10.0 ml portions of 0.05 *M* Tris buffer at pH 8.6. Again, because of the small yield, Fractions 7 and 6 were combined. About 5–10% of the total RNA remained in the phenolic phase, which could be extracted only with such KOH solutions that considerable degradation of the RNA occurred.

The RNA in the extracts was precipitated with 95% ethanol and, after standing in the cold overnight, was centrifuged. No DNA could be detected in any of the extracts. The RNA precipitates were dissolved in Tris

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TABLE I. Distribution of RNA Extracted from Nuclei in Regenerating Wound Tissue.

Fraction No.	pH	Days after wounding[a] 5 RNA (μmoles/mg of DNA)	%	8 RNA (μmoles/mg of DNA)	%	12 RNA (μmoles/mg of DNA)	%
1	7.2	91.2 ± 3.8	60	190.0 ± 8.5	61	111.8 ± 11.4	55
2	7.2	22.5 ± 2.7	15	46.8 ± 5.6	15	41.4 ± 4.4	19
3–4	7.2	20.9 ± 2.3	14	26.8 ± 2.8	9	25.5 ± 2.1	12
5	8.6	7.1 ± 2.0	5	23.2 ± 2.5	8	13.8 ± 1.5	6
6–7	8.6	9.3 ± 2.1	6	22.1 ± 1.4	7	18.6 ± 2.3	9
Nuclear RNA		151.0		308.9		211.1	
Total RNA[b]		540		720		630	
Cytoplasmic RNA		389		411		419	

[a] Data for each experimental day was obtained from the analysis of 4 pools of 20 samples of regenerated tissue.

[b] Calculated from data in (5).

buffer (pH 7.2) and the absorption was measured between 220–300 mμ in a Cary-15 recording spectrophotometer. The RNA was reprecipitated with ethanol and hydrolyzed in 0.1 *M* NaOH at 37° for 18 hr (11). The resulting nucleotides were separated on a Dowex 50W-X4 column, and their concentration was determined spectrophotometrically, by the method reported by Katz and Comb (12). From this data, the base ratio of each extract was calculated. The DNA in the residual phenolic phase, from which the RNA had been extracted, was precipitated with diethyl ether and measured with diphenylamine (14).

To measure the rate of protein synthesis, weighed wound tissue samples were homogenized in 5% trichloroacetic acid and the precipitate hydrolyzed in 6 *N* HCl at 120° for 20 hr. After oxidation with chloramine T, the proline derivative was extracted into toluene. The oxidized hydroxyproline in the residual aqueous phase was decarboxylated at pH 8.3 by heating in a boiling water bath, and then extracted into another portion of toluene. Radioactivity in aliquots of both toluene extracts was measured by liquid scintillation counting. The amount of proline derivatives was determined with Ninhydrin in glacial acetic acid; the level of hydroxyproline was measured by reaction with *p* dimethylaminobenzaldehyde (14). The first toluene extract contained no hydroxyproline derivative; the second, no proline derivative.

Results and Discussion. The level of RNA in the nuclei of granulation tissue at different stages of regeneration was determined (Table I) by adding the amount of the nucleotides found in each extract. The nuclear RNA per cell appears to increase rapidly to a maximal level in about 8 days and then begins to decline. A comparison with the total RNA in the cell, calculated from previously published data, is also shown in Table I (5). As shown, the changes in RNA content as the tissue regeneration proceeds are due largely to changes in the amount of RNA in the nuclei.

The relative rate of the production of collagen can be deduced from the appearance of ^{3}H-hydroxyproline per hour in the tissue (Fig. 1). By subtracting the theoretical ^{3}H-proline content of the collagen from the total ^{3}H-proline measured in the tissue, we could calculate the amount of ^{3}H-proline incorporated into the cellular proteins. The relative rate of synthesis of cellular proteins could then be plotted. The plots in Fig. 1, show that the relative rates of both cellular protein and collagen synthesis in this tissue parallel the changes of RNA level in the nuclei.

A preliminary indication of whether there are qualitative changes in the nuclear RNA as the wound tissue matures, was obtained from the absorption spectra of the various

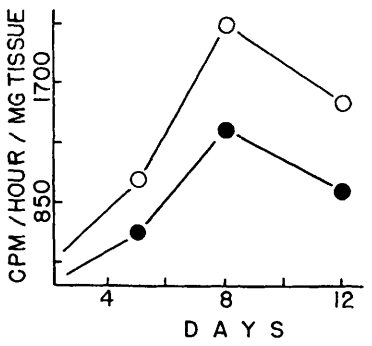

FIG. 1. The relative rate of synthesis of proteins in granulation tissue at different stages of regeneration plotted as labeled amino acid incorporated per hour per milligram of tissue against days after wounding. The tissue was removed for analysis 3 hr after administration of 45 μCi of 5-^{3}H-L-proline. (●) activity of ^{3}H-hydroxyproline in the tissue; (○) the ^{3}H-proline in the cellular proteins. The labeled proline in the cellular proteins was calculated by subtracting the theoretical amount of labeled proline in the collagen from the total ^{3}H-proline in the tissue. It was assumed that collagen contains 12.5% hydroxyproline and 14% proline.

fractions (Fig. 2). The spectra of Fractions 1 (Fig. 2a), representing some 50–60% of the total nuclear RNA, suggest that most of the RNA moieties are unchanged during the period of regeneration studied. The similarity of the spectra of the first and second neutral extracts points to the possibility that much of the RNA in both may be similar. Again, the spectra of the RNA in the second extract (Fig. 2b) are practically unchanged as tissue regeneration progresses. These data suggest that during the course of wound tissue formation most of the RNA in the nuclei remains the same. However, the spectra of the RNA in the other extracts (Fig. 2c, d, e), representing some 25% of the total, indicate a significant change in base composition. The shift of the absorption maxima of these spectra to longer wavelengths at neutral pH implies the appearance of RNA with a relative decreased proportion of cytidylic and guanylic acid residues as tissue regeneration proceeds.

To verify these qualitative changes in the RNA population of the nuclei, nucleotide analysis of the RNA extracted was carried out. The data in Table II show that in agreement with the spectral data the ratio of nucleotide bases in the RNA in Fration 1 remains virtually unchanged during tissue regeneration. Fraction 1 is composed mainly of RNA having a high (G + C)/(A + U) ratio. RNA moieties in Fraction 2 are about the same as those in Fraction 1 on the fifth day after wounding. However, a gradual replacement of RNA with a lower (G + C)/(A + U) ratio occurs with time. The RNA molecules in the Fractions 3–4 change radically during the experimental period; the ratio of (G + C)/(A + U) changes from a relatively high to a relatively low value, presumably as the result of a drastic increase in proportion of the DNA-like RNA described by Georgiev (15). RNA in Fractions 5 and 6–7 appear to consist mainly of this DNA-like RNA with a relatively low (G + C)/(A + U) ratio.

Judging from the spectra and the nucleotide analysis data, nuclear RNA metabolism in the wound tissue seems to entail a steady increase of DNA-like RNA having a low (G + C)/(A + U) ratio, as regeneration proceeds. The greatest increase in the proportion, as well as in the absolute amount, of this low (G + C)/(A + U) type(s) of RNA occurs between 5 and 8 days after wounding (Tables I and II), paralleling the time when the greatest increase in the rate of protein synthesis is observed (Fig. 1). The absolute amount of RNA with a high (G + C)/(A + U) ratio (Fractions 1 and 2) doubles from the fifth to the eighth day of tissue regeneration; thereafter the amount of such types of RNA decrease markedly.

TABLE II. Ratio of Bases in Nuclear RNA Fractions of Regenerating Wound Tissue.

Fraction no.	(G + C)/(A + U) Days after wounding: 5	8	12
1	1.5	1.6	1.6
2	1.5	1.3	1.1
3–4	1.4	0.5	0.5
5	0.9	0.5	0.5
6–7	1.0	0.5	0.4
	RNA (μmoles/mg of DNA)		
Ratio			
≧1.1	134.6	236.8	153.2
≦1.0	16.4	72.1	57.9

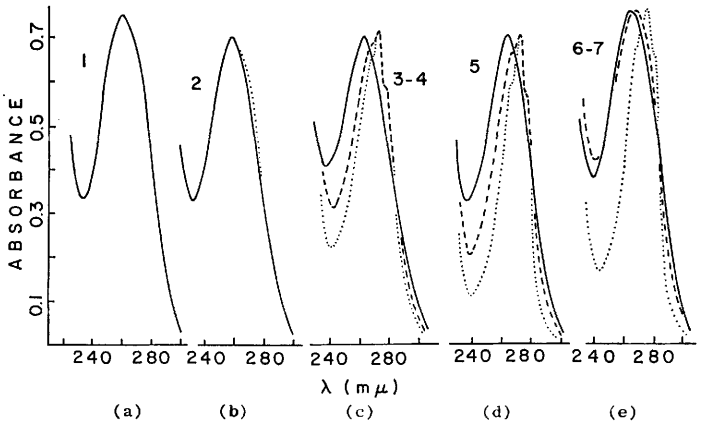

FIG. 2. Absorption spectra of the RNA extracted from the nuclei of granulation tissue at different stages of regeneration. (—) the spectrum of the nuclear RNA obtained 5 days after wounding; (- - -), 8 days; (. . .), 12 days. (a) Fraction 1, first neutral pH extract; (b) Fraction 2, second neutral extract; (c) Fraction 3–4, combined third and fourth neutral extracts; (d) Fraction 5, first extract at pH 8.6; (e) Fraction 6–7, combined second and third extracts at pH 8.6. In (a) and (b) the three spectra are so similar that only a single curve is apparent.

These quantitative and qualitative changes in the nuclear RNA probably indicate that the template activity in the cells of regenerating tissue varies considerably during the course of wound tissue formation. The quantitative variations in nuclear RNA, possibly may be due to the presence of different levels of RNA polymerase or ribonuclease, or both. The appearance of different types of nuclear RNA during tissue regeneration probably is the result of derepression, followed by transcription, of new and different genomes on DNA, possibly by the mechanisms of acetylation, phosphorylation, and/or methylation of the histones (16). The data also seem to suggest that a direct relation exists between the amount of nuclear RNA and the rate of protein synthesis observed in regenerating wound tissue.

Summary. Previously reported changes of total RNA in the cells of regenerating wound tissue are largely accounted for by changes in the level of nuclear RNA. The rates of collagen and cellular protein syntheis in this tissue coincide with the quantitative changes in the level of nuclear RNA. As wound tissue regeneration progresses, there is a marked increase in the proportion of DNA-like RNA in the nuclei.

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