Failure of Cultured Chick Embryo Fibroblasts to Incorporate Collagen into Their Extracellular Matrix When Transformed by Rous Sarcoma Virus

AN EFFECT OF TRANSFORMATION BUT NOT OF VIRUS PRODUCTION

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Whole chick embryo fibroblasts were infected with the Prague wild type Rous sarcoma virus and with a temperature-sensitive mutant of this strain, RSVtsLA24. Normal fibroblasts and fibroblasts infected with the temperature-sensitive mutant and cultured at the nonpermissive temperature, secreted procollagen into the medium and incorporated collagen into their extracellular matrix. On the other hand, transformed fibroblasts and fibroblasts infected with the temperature-sensitive mutant and cultured at the permissive temperature, were able to secrete procollagen into the medium, but there was no evidence that they were able to convert procollagen to collagen and incorporate collagen into their extracellular matrix. This inability of the infected cells to incorporate collagen into an extracellular matrix was found to be a result of transformation rather than of virus production in these cells.

Interstitial collagen is composed of three peptide chains with a molecular weight of 95,000 each. It is synthesized as a larger molecule (molecular weight 130,000/peptide), procollagen (Jimenez et al., 1971; Layman et al., 1971; Ballamy and Bornstein, 1971), which is converted to collagen by a number of proteolytic cleavages (for review, see Martin et al., 1975). Procollagen, but not collagen, from fibroblasts contains interchain disulfide crosslinks (Layman et al., 1971; Smith et al., 1972; Goldberg et al., 1972; Dohm et al., 1972; Burgeson et al., 1972) which are distinguishable from lysine and hydroxylysine-derived cross-links. The latter can be prevented from forming by L-aminopropionitrile, an inhibitor of lysyl oxidase (Pinnell and Martin, 1968). Procollagen is transported to the plasma membrane by the microtubule system (Ehrlich and Bornstein, 1972; Dehm and Prockop, 1972; Diegelman and Peterkofsky, 1972) where it can either be secreted into the medium and remain as procollagen, or it can be cleaved to collagen and incorporated into the extracellular matrix (Layman et al., 1971).

When cultured chick embryo fibroblasts are transformed by Rous sarcoma virus (RSV), there is a decrease in the amount of collagenous protein synthesized (Levinson et al., 1975). The decrease in collagen biosynthesis is accompanied by alterations in cell morphology (Terui, 1960), surface change (Patrick et al., 1970), and contact behavior (Levinson et al., 1971). While Hakomori (Hakomori et al., 1971; Hakomori, 1973) has suggested that the altered surface properties may be related to biochemical changes in the glycoprotein and glycolipid composition of the cell membrane, Warren et al. (1972) have produced evidence to refute this speculation. An increase in hyaluronic acid production (Ishimotra et al., 1966; Okayama et al., 1977) has also been reported to occur upon transformation. In this paper, the authors have characterized the collagen synthesized by the transformed cells in an attempt to understand the altered characteristics of these cells.

MATERIALS AND METHODS

Viruses and Cells—The RSV strains used in this study were the Prague strain wild type and a temperature-sensitive mutant of this strain. The mutant RSVtsLA24 has been isolated by Wyke and Lineal (1973) and belongs to the class T mutant (Wyke, 1974). The temperature-sensitive lesion is at the sar gene, thus allowing replication of the virus to take place normally at the nonpermissive temperature (41°C). Primary chick embryo (eggs obtained from SPA-FAS Co., West Chester, Pa.) cell cultures were obtained as previously described (Vogt, 1969). Secondary cultures were obtained by seeding 2 x 10⁵ cells into 10-cm plastic Petri dishes and incubating at 37°C in a humidified air/5% CO₂ atmosphere. The culture medium was F10 (Ham, 1963) containing 5% fetal calf serum, 1% chicken serum, and 1% dimethylsulfoxide. Secondary cultures were grown and infected with RSV (multiplicity of infection was approximately 40 U/S.C. Section 1784 solely to indicate this fact.

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The abbreviations used are: RSV, Rous sarcoma virus; SDS, sodium dodecyl sulfate.
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**RESULTS**

The incorporation of \(^{3}H\)proline into total protein and the synthesis of hydroxy\(^{3}H\)proline by transformed and normal fibroblasts are shown in Table I. Both the infected and normal cells incorporate \(^{3}H\)proline into nondialyzable material at comparable rates when the data are calculated per mg of protein, indicating that the infected and normal cells have comparable rates of synthesis. However, of the radioactive proline incorporated into nondialyzable material, a greater portion is secreted into the media by the normal cells than by the transformed cells. RSV-infected cells synthesize less collagen than normal cells as indicated by radioactive hydroxy\(^{3}H\)proline incorporation (Table I, column 4). The purification enriched the radioactive hydroxyproline containing protein in the normal cell fraction up to detectable levels, whereas no hydroxy\(^{3}H\)proline was found to be associated

| Table I | Incorporation of \(^{3}H\)proline and synthesis of hydroxy\(^{3}H\)proline in normal and transformed cells and culture medium of normal and transformed cells in typical experiment |
|--------------------------|-------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
|                         | \(10^5 \times \text{[H]}\) cpm | \(10^5 \times \text{[H]}\) cpm | \(10^5 \times \text{hydroxy[H]}\) cpm | Per cent hydroxy\(^{3}H\)proline in total cpm |
|                         | dish                         | mg protein                                     | \(\mu g\) DNA                                | (total cpm 100)                                 |
| Starting material       |                               |                                               |                                               |                                                  |
| Normal cells            | 25.5                          | 21.0                                          | 1.56                                          | N.D.                                           |
| Medium of normal cells  | 3.2                           |                                               | 40.0                                         | N.D.                                           |
| Transformed cells       | 60.7                          | 23.3                                          | 0.877                                        | N.D.                                           |
| Medium of transformed cells | 2.0                         |                                               | 1.7                                          | N.D.                                           |
| Partially purified collagen fraction |                      |                                               |                                               |                                                  |
| Normal cells            | 5.0                           | 28.5                                          | 11.4                                         | 4.3                                             |
| Medium of normal cells  | 2.6                           |                                               | 35.3                                         | 25.1                                            |
| Transformed cells       | 8.0                           | 19.6                                          | N.D.                                         | N.D.                                           |
| Medium of transformed cells | 1.1                         |                                               | 1.4                                          | 8.8                                             |

* N.D. Not detectable (less than 0.3% of total counts per min was hydroxyproline and this much was due to spillover from proline).
with the transformed cells even after this enrichment.

From the data in Table I, it can be seen that the normal cells have at least 10 times more collagen on a percentage basis than the transformed cells (4.3% versus <0.3%) and at least 23 times more collagen than the transformed cells on a DNA basis.

**Normal Cell Fraction** – Labeled, partially purified collagen from the normal cell layer showed two major peaks on CM-cellulose chromatography at Fractions 84 and 108 (Fig. 1A). Analysis of these fractions showed that hydroxy[¹⁴C]proline constituted 35 and 28%, respectively, of the total [¹⁴C], indicating that the peaks are collagenous. The void volume peak was found not to contain radioactive hydroxyproline thus categorizing it as noncollagenous. Recovery of total counts from CM-cellulose was 43 to 58%. The SDS disc gel electrophoretic pattern of the normal cell fraction showed two peaks migrating closely with the \( \alpha_1 \) and \( \alpha_2 \) chains of calf-skin collagen standards (Fig. 1B). Only 8% of this radioactive sample contained reducible cross-links, suggesting that the peaks are collagen rather than procollagen. Recovery of counts from the gels ranged between 51 to 68%. Because of its elution from CM-cellulose and its migration on SDS-polyacrylamide disc gels, the radioactive hydroxyproline containing material isolated from the normal cell layer was identified as type I collagen. This is in agreement with work done on human fibroblasts (Goldberg and Sherr, 1973).

**Culture Medium Fraction of Normal Cells** – The major collagenous component, obtained from the medium of normal cells was found to elute after \( \alpha_2 \) on CM-cellulose (Fig. 2A). Fraction 119 was found to contain 30% radioactive hydroxy[¹⁴C]proline. When the material was electrophoresed on SDS-polyacrylamide disc gels the major portion migrated between \( \beta_1 \) and \( \alpha_1 \) standards (Fig. 2B) and contained 41% of the counts as reducibly cross-linked material, indicating that it represents procollagen rather than collagen.

**Transformed Cell Fraction** – The CM-cellulose pattern from the virus transformed cell layer contained a major peak at the void volume of the column (Fig. 3A). Upon analysis, the void volume material was found to contain less than 0.3% radioactive hydroxy[¹⁴C]proline, indicating that it was not collagenous. Material which eluted in the vicinity of the collagen standards, fractions 84 and 125, was also found to contain no radioactive hydroxyproline. There is also no prominent peak seen in the collagen region when the sample is electrophoresed on SDS polyacrylamide disc gels (Fig. 3B). Only 6% of the material is reducibly cross-linked, but these counts are not shifted to the procollagen region upon reduction. There are thus no significant peaks associated with the transformed cells which can be characterized as collagenous by either CM-cellulose or SDS-polyacrylamide disc gel electrophoresis. The standard collagen purification did not lead to the isolation of either a collagen or procollagen type molecule from the transformed cell fraction. By using the radioactive hydroxyproline value at fractions 84 and 125 (Fig. 3A) and assuming that any collagen in the transformed cell sample would elute from CM-cellulose in the same position as collagen from the normal cell sample, it can be calculated that the maximum amount of hydroxyproline in the transformed cell fraction is 700 cpm/500,000 cpm of CM-cellulose sample. When this data is expressed on a DNA basis there is, at most, 10 hydroxy[¹⁴C]proline cpm/µg of DNA associated with the transformed cell fraction. There were 3500 hydroxy[¹⁴C]proline cpm/µg of DNA associated with the normal cell fraction (Fig. 1A), a 550-fold difference in the collagen of these two samples.

To exclude the possibility that there was collagen associated with the transformed cell fraction but that it was degraded during isolation by an enzyme present in the virus-infected cells, a protease inhibitor mixture was included in one-half of the sample during one of the preparations. The preparations with the inhibitor mixture gave identical patterns to those without the inhibitor mixture on SDS disc gel electrophoresis, indicating that the collagen, if it was present, was not being degraded during preparation by any of the proteases usually present.

**Culture Medium Fraction of Transformed Cells** – The labeled, partially purified material isolated from the medium of transformed cells shows multiple peaks on CM-cellulose (Fig. 4A). The radioactive material passing directly through the column contained no detectable radioactive hydroxyproline. The peak at Fraction 62 contains 3% hydroxy[¹⁴C]proline; Fraction 78, 11%; Fraction 93, 13%; and Fractions 113 and 119, 20% hydroxy[¹⁴C]proline. On SDS-polyacrylamide disc gel electrophoresis in the absence of 2-mercaptoethanol, 29% of the counts remain at the origin. Upon reduction these counts are shifted to the region between \( \beta_1 \) and \( \alpha_1 \) (Fig. 4B), indicating that the radioactivity represents procollagen. As shown in Fig. 4B, the medium of transformed cells has a collagenous component with electrophoretic properties similar...
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Evidence That Change of Collagen upon Transformation Is Related to Viral Transforming Gene Product — Since infection with the wild type RSV causes both virus replication and transformation at the same time, the effect of RSV on the collagen production by these cells could simply be due to the production of virus and not to the fact that they are transformed. To establish this point, cells infected with RSVtsLA24 were studied. This mutant has a temperature-sensitive lesion in its transforming gene but viral replication goes on normally at the non-permissive (41°C) as well as at the permissive (37°C) temperature.

The cells, infected with the RSV temperature-sensitive mutant and cultured at 37°C, synthesized collagenous protein similar to the wild type infected cells. On SDS polyacrylamide disc gel electrophoresis there was no collagen associated with the cell layer (Fig. 5A), while a procollagen-like protein was found in the medium (Fig. 5B), much the same as from the cells and medium of cells infected with the wild type RSV (compare Figs. 5A and B with Figs. 3B and 4B, respectively). When cells infected with the temperature-sensitive mutant were cultured at the nonpermissive temperature, 41°C, they resembled the normal cells with respect to their collagen biosynthesis. Collagen was found to be associated with the cell fraction (Fig. 6A) and procollagen with the medium fraction (Fig. 6B). Normal cells cultured at 41°C produced collagen and procollagen similar to normal cultures at 37°C (data not shown).

Discussion

In the normal cultures the fibroblasts incorporate radioactive proline into procollagen, part of which is secreted into the medium and part of which is converted to collagen and incorporated into the extracellular matrix. This interpretation is based on the CM-cellulose and SDS-disc gel electrophoresis results (Figs. 1, A and B, and 2, A and B) in this paper and agrees with the results obtained by others (Layman et al., 1971; Goldberg and Sherr, 1973). The approximate 2:1 ratio of α1 to α2 components characterizes this material as a type I collagen.

Although there are some differences in the radioactive material isolated from the medium of normal cells compared
with the medium of transformed cells, the overall pattern is similar (compare Fig. 2A with Fig. 4A and Fig. 2B with Fig. 4B). The transformed cells are synthesizing and secreting a radioactive material similar to the radioactive procollagen seen in the media of normal cells. However, while the transformed cells are synthesizing and secreting procollagen, there appears to be no incorporation of collagen into their extracellular matrix. No radioactive collagen peaks are apparent on either CM-cellulose (Fig. 3A) or SDS-polyacrylamide disc gel electrophoresis (Fig. 3B) and no (<0.3%) radioactive hydroxyproline was detectable in the purified fraction (Table I).

The transformed cultures are synthesizing less collagenous material than the normal cultures (Table I) as was also demonstrated by Levinson et al. (1973b). By taking the normal cell fraction through a collagen purification process the radioactive hydroxyproline is increased to 4.3% of the total counts, whereas no detectable hydroxyproline counts appear in the fraction purified from the transformed cells. Even though the transformed cultures are making less collagen than the normal cultures, if there were hydroxyproline containing material in the transformed cell fraction, similar to that in the normal cell fraction, it would have been detected by at least one of the techniques used in this investigation. The conclusion is then that there is essentially no collagen associated with the RSV transformed cell matrix.

There are several possible explanations for the absence of collagen in the transformed cell matrix. One possibility is that the procollagen synthesized by the transformed cells is altered in some way so that it does not act as a substrate for procollagen peptidase. Whether the differences in the CM-cellulose (Fig. 2A and Fig. 4A) and SDS-disc gel electrophoresis patterns (Fig. 2B and Fig. 4B) and the difference in the percentage of hydroxyproline (30% for the major medium peak of the normal cells versus 20% for the major medium peak of the transformed cells) in the radioactive material from the medium of normal cells, compared with the medium of transformed cells represents actual differences in the procollagen or the presence of contaminating noncollagenous proteins cannot be determined in the present data. Because of the elevated levels of prolyl hydroxylase in RSV transformed chick embryo fibroblasts, Levinson et al. (1975) concluded that the collagen synthesized by these cells was fully hydroxylated and thus the difference in hydroxyproline values observed in this study would be due to contaminating noncollagenous proteins. However, increased prolyl hydroxylase levels do not necessarily guarantee fully hydroxylated procollagen. The fact that the procollagen from the medium of transformed and normal cells gives similar patterns upon electrophoresis and chromatography would indicate that if there were an alteration in procollagen it would of necessity have to be minor.

Another possible explanation for the absence of collagen in the cell matrix of the transformed cells would be an increase in the level of proteases associated with transformation. The results of the experiment where the inhibitors were maintained during the purification of the collagen would suggest that there is no degradation occurring during isolation. If the collagen is being degraded then it must be degraded while the cells are in culture. Increased levels of collagenase have been associated with carcinoma cells in vivo but the collagenase activity was not demonstrable when the cells were cultured in vitro (Harris et al., 1972). Another argument against collagen being degraded after conversion from procollagen is the presence of a large amount of serum in the medium. The serum present in the culture medium would be more than sufficient to inhibit any collagenase produced by the transformed cells. Therefore, if collagen is produced by procollagen peptidase which is shown to be present in the medium (Layman and Ross, 1978), it should remain intact even in the transformed cell cultures. In addition, when radioactive procollagen, isolated from the medium of normal cells, was incubated with cultures of transformed or normal cells, for periods up to 24 h, there was no appreciable conversion of radioactive procollagen, which is a substrate for collagenase, to a lower molecular weight and there was no difference between the transformed and normal cultures. Neither the transformed nor the normal cells incorporated the radioactive procollagen into their extracellular matrix or cell layer. It is therefore unlikely that the extracellular conversion of procollagen to collagen occurs normally with the collagen being subsequently degraded by an extracellular collagenase in the transformed cultures.

A third possible explanation for the absence of collagen in the transformed cell matrix is that the conversion of procollagen to collagen is somehow prevented. The changes in the cell membrane glycolipids and glycoproteins of RSV transformed cells (Hakomori et al., 1971; Hakomori, 1973) may affect events involved in the alignment of individual pro α chains and triple helix formation. A block in any of these steps would severely inhibit procollagen conversion to collagen and thus incorporation of collagen into the extracellular matrix. Another necessary factor for the conversion of procollagen to collagen is procollagen peptidase. If the activity of this enzyme were absent, conversion would not occur. Procollagen peptidase activity has been demonstrated by Levinson et al. (1975). A third possibility is that the procollagen synthesized by the transformed cells is not degraded by an extracellular collagenase.

Unpublished data.
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Collagen peptidase was found to be absent in the medium of human fibroblasts during logarithmic growth and its activity was not detectable in these cells during any stage of growth (Layman and Ross, 1973). Because the transformed cells are not contact inhibited, their continued growth may prevent the synthesis of procollagen peptidase. The control cultures are confluent when incubated with the radioactive proline and therefore would be expected to be secreting procollagen peptidase into the medium. Of the three possible explanations for the absence of collagen in the extracellular matrix of transformed cells, the latter involving the absence of procollagen peptidase seems, at this time, to be the most plausible. The precise explanation must, however, await further experimentation.

Since virus production occurs at both the permissive and nonpermissive temperatures in cells infected with RSVtsLA24, it is possible to distinguish the effects of virus production from those of transformation. The observation that infected cells incorporate collagen into their extracellular matrix at the nonpermissive temperature (Fig. 6, A and B) demonstrates that virus production does not interfere with collagen processing. The block in procollagen processing seen in the infected cells at the permissive temperature (Fig. 5, A and B) must therefore be a result of transformation alone and not virus production. The change of morphological and biochemical characteristics occurs relatively fast (within 24 h) upon raising the temperature of the RSVtsLA24 infected cells from 37° to the nonpermissive temperature (41°), indicating that this effect is probably not due to the selection of different cell populations.

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