Regulation of Collagen Gene Expression by Transformed Human Fibroblasts: Decreased Type I and Type III Collagen RNA Transcription

Atsushi Hatamochi, Masashi Ono, Hiroaki Ueki, and Masayoshi Namba
Department of Dermatology (AH, MO, HU), Kawasaki Medical School, Kurashiki; and Department of Pathology (MN), Institute of Cancer Research, Okayama University Medical School, Okayama, Japan

The regulation of collagen gene expression in normal diploid human fetal fibroblasts (KMS-6 cells), and fibroblasts immortally transformed by treatment of KMS-6 with Co-60 gamma rays (KMS-6 cells) was compared to that of ones tumorigenically transformed by treatment of KMS-6 cells with Harvey murine sarcoma virus (KMS-6-Ras cells). Synthesized collagenous protein decreased to approximately 30% of that of normal fetal fibroblasts in both transformed cell lines, and the relative rate of collagen synthesis to total protein synthesis decreased about sixfold in KMS-6 cells and twelvefold in KMS-6-Ras cells. The m-RNA levels of type I collagen in both of these cell lines decreased to approximately 20% of that of the control fibroblasts, whereas type III collagen m-RNA levels decreased to only 9% of that of the control. The copy number of the collagen gene in both transformed cell lines was unaltered. The transcriptional rates of collagen α1(I) and collagen α1(III) in both cell lines decreased to 20% and 7% respectively of that of control. These data indicate that collagen synthesis was reduced at the transcriptional level in these transformed human fibroblasts. J Invest Dermatol 96:473–477, 1991

The biosynthetic patterns of certain cellular proteins change in the process of malignant transformation of cells. The rate of synthesis increases for some proteins and decreases for others [1–4]. There have been many reports of decreased collagen synthesis in chemically or virally transformed fibroblasts. These studies have been done mainly on animal cells, such as mouse fibroblasts [5–11].

Correct regulation of collagen gene expression is a prerequisite for the normal functioning of connective tissue. Studies of the mechanisms that modulate the gene expression of collagen in human fibroblasts should provide a better understanding of the regulation of connective tissue metabolism in human skin, and investigating collagen gene expression in transformed human fibroblasts is one approach to the study of the regulation of that metabolism. Decreased collagen synthesis in SV40-transformed human fibroblasts has been reported by many authors [12–15]. However, collagen synthesis in other types of transformed human fibroblasts has not been sufficiently studied.

Recently, we established multistep carcinogenesis of fetal human fibroblasts [13]. Normal diploid human fetal fibroblasts were transformed into an immortal cell line by treatment with Co-60 gamma rays, and the cells of this immortal cell line were then converted into neoplastic cells by treatment with Harvey murine sarcoma virus. In the present report, we describe a comparison of the regulation of collagen gene expression in these normal and transformed human fibroblasts.

MATERIALS AND METHODS

Cell Lines and Cell Cultures The cell lines used were as follows: 1) KMS-6, normal diploid human fetal fibroblasts cultured from a 9-week-old whole embryo in our laboratory; 2) KMS-6, fibroblasts transformed immortally by treatment of KMS-6 cells with Co-60 gamma rays, and 3) KMS-6-Ras, fibroblasts transformed neoplastically by treatment of KMS-6 cells with Harvey murine sarcoma virus. The details of the procedure for the transformation of cells and their cellular characteristics have been previously reported elsewhere [16]. All of these cells were cultured in Eagle’s minimum essential medium (Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal calf serum. The cells were subcultured using 0.2% trypsin solution (1:250; Difco Laboratories, Detroit, MI) in phosphate-buffered saline (PBS).

Measurement of Collagen Synthesis Cells inoculated onto 35 × 10 mm plastic dishes were assayed at confluency. The culture medium was changed to Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 50 μg/ml ascorbic acid and 50 μg/ml β-aminopropionitrile containing 5 μCl/ml L-[2,3-3H] proline (specific activity 27.7 Ci/mmol, NEN Chemicals, Boston, MA) and incubated in a CO₂ incubator for 3 h at 37°C. The protein concentrations per dish were assayed with the Protein assay kit (Bio-Rad, Richmond) [17]. After labeling, 0.5 mM phenylmethylsulfonyl flu-
oride was added to the cultures and the medium was collected. Cell layers were washed with 2 ml of cold phosphate-buffered saline (PBS) and harvested. The medium and the cells were mixed and then sonicated. Collagen synthesis was assayed by measuring the radioactivity of the medium and cells together after limited digestion with purified bacterial collagenase, according to the method of Peterlik and Dinehart [18] with minor modifications. Briefly, in the presence of 50 μg/ml of bovine serum albumin as a carrier protein, trichloroacetic acid (TCA) was added to the assay sample in a final concentration of 10% and the suspension was kept at 0°C for 5 min. After the precipitate was collected by centrifugation at 1000 × g for 5 min, it was resuspended with 1.0 ml of 5% TCA containing 1 mM proline and then recentrifuged. The supernatant fraction was then removed. These procedures were repeated two more times. The precipitates were dissolved and neutralized in 0.05 M NaOH, digested with collagenase form III (Advance Biofactures Corporation, Lynbrook, NY) and divided into collagenase-sensitive protein (CSP) and non-collagenase–sensitive protein (NCSP) samples. Then the radioactivity of these samples was counted in a liquid scintillation spectrometer. After correction for the protein concentration of the cell layer in each dish, these values were considered as the collagenous protein synthesis and non-sensitive collagenous protein synthesis values. The relative rate of collagen synthesis to total protein synthesis was calculated with the assumption that collagen has an amino acid content 5.4 times higher than that of other proteins [19].

Determination of m-RNA Levels When the cell layers incubated onto 150 × 10 mm dishes achieved confluency, the medium was removed and the cells were washed twice with cold PBS. To obtain the total cellular extract, 5 M guanidine thiocyanate containing 0.75% of 2-mercaptoethanol was added to the dishes, and total RNA was isolated by centrifugation over a cushion of 5.7 M cesium chloride [20]. RNA (1 μg/lane) was separated by electrophoresis on 1% agarose/formaldehyde gels, after which it was transferred to a nitrocellulose filter for Northern hybridization by standard procedures [21]. For dot blot analysis, RNA (5–0.3125 μg) was denatured in a buffer containing formaldehyde for 15 min at 65°C and applied to nitrocellulose filters using a vacuum dot blot template. Filters were then baked for 2 h at 80°C under a vacuum and prehybridized for 3 h at 42°C in 10 × Denhardt’s solution containing 50% formamide 0.75 M NaCl and 0.075 M sodium citrate (5 × SSC), 0.05 M phosphate (pH 6.5) and sonicated salmon sperm DNA (250 μg/ml). Specific hybridization was carried out for 24 h at 42°C as described elsewhere [22] with 32P-labeled cDNA probes specific for collagen α(I), collagen α(III), and β-actin. The filters were washed twice in 2 × SSC, 0.1% SDS at room temperature, twice in 0.1 × SSC, 0.1% SDS at 62°C, air dried and exposed to x-ray films at −80°C for various periods of time. The radioactivity was determined by scanning autoradiograms with a densitometer. Plasmid Hf-677 with a 1.5-kilobase (kb) insert specific for the α(I) collagen chain [23], plasmid pHII-33 with a 0.9-kb insert for the α(I) collagen chain [24], and plasmid pHFA-1 with a 0.5-kb insert for β-actin [25] were used as DNA probes for collagen I, III, and β-actin m-RNA, respectively.

DNA Preparations and Southern Blot Analysis High molecular weight DNA was extracted as described by Wilger et al [26]. Purified DNA was digested by restriction enzymes, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose by the method of Southern [27]. Hybridization was carried out as described above, and filters were washed twice in 2 × SSC with 0.1% SDS at room temperature, followed by two washes in 0.1 × SSC with 0.1% SDS at 55°C. The DNA fragments described above were used as DNA probes.

Nuclear Run-Off Assay Three days after incubation of each cell line, 80% confluent cells were harvested and washed twice with PBS; nuclei were then isolated with NP-40 by the procedure of Groudine et al [28] and stored at −70°C. For transcription with nuclease, the nuclei were incubated with 100 μl of a reaction buffer [16% glycerol, 20 mM HEPES pH 8.0, 0.04 M EDTA, 5 mM MgCl2, 0.5 mM MnCl2, 0.4 mM each of adenosine 5′-triphosphate (ATP), cytidine 5′-triphosphate (CTP), and uridine 5′-triphosphate (UTP) and 100 μCi of guanosine 5′-[α-32P]triphosphate ([α-32P]-GTP) for 45 min at 25°C [29]. The 32P-labeled RNA was purified by the method of Tullis and Rubin [30] and was hybridized with plasmid DNA immobilized on nitrocellulose filters. Four and two micrograms of each DNA sample were denatured by incubation with 0.3 M NaOH for 30 min at 65°C, neutralized by adding 1.0 volume of 2 M ammonium acetate, and then applied to nitrocellulose filters on a slot blot template. Each filter was hybridized with the same number of counts per minute of the 32P-labeled RNA. After hybridization, the filters were incubated in 2 × SSC, containing 0.1% sodium dodecyl sulfate (SDS) at room temperature and then in 0.1 × SSC, with 0.1% SDS for 30 min at 65°C. RNAase A treatment of the filters at 10 μg/ml in 2 × SSC (37°C, 20 min) reduced the background by removing overhanging RNA tails. The filters were exposed to Kodak XAR-5 film with a DuPont Cronex Lightning Plus intensifying screen for various time periods.

Statistical Analysis The statistical significance of the data was calculated using the Student t test.

RESULTS

Collagenous and Non-collagenous Protein Synthesis of Transformed Human Fibroblasts Table I shows the collagen synthesis and non-collagenous protein synthesis of each cell line. The synthesis of collagen was calculated as the percentage of the total protein synthesis. The collagen synthesis in both transformed cell lines was about 3 times lower than that in normal fetal fibroblasts. The non-collagenous protein synthesis in KMST-6 and KMST-6-Ras cells was approximately 2 times and 4 times higher, respectively, than that in KMS-6 cells. Therefore, approximately fivefold and tenfold reductions of collagen synthesis to total protein synthesis were observed in KMST-6 and KMST-6-Ras cells, respectively.

Steady-State Level of α(I) and α(III) Collagen m-RNA To determine whether the reduction of collagen synthesis in both transformed cell lines was due to changes in the steady-state level of collagen mRNA, we performed Northern blot analysis using total RNA from each cell line. The α(I) collagen-specific cDNA probe hybridized to two distinct m-RNA species with apparent sizes of 5.8 and 4.8 kb. The densitometric values of these bands were reduced in KMST-6 and KMST-6-Ras cells (Fig 1A). The α(III) collagen-specific cDNA probe revealed two major m-RNA bands with apparent sizes of 5.4 and 4.8 kb. The densitometric values of these bands were also significantly reduced in KMST-6 and KMST-6-Ras cells (Fig 1B). On the other hand, the level of β-actin m-RNA was almost the same (Fig 1C). For a better quantification, the steady-state levels of specific m-RNA coding for different proteins were determined by dot blot analysis (Fig 2). Although the values of β-actin m-RNA were not altered following transformation of the cells (Table II), both transformed cell lines showed an approximate fivefold decrease in α(I) collagen m-RNA and a tenfold decrease in α(III) collagen m-RNA compared to normal fetal fibroblasts (Table II).

| Table I. Collagen Synthesis by Transformed Fibroblasts |
|-----------------|-----------------|-----------------|
| Cells           | CSPa (dpm/μg protein) | NCSPa (dpm/μg protein) | Ratio of Synthesis of Collagen to Total Proteinb |
| KMST-6          | 216 ± 25        | 287 ± 24        | 12.2           |
| KMST-6-Ras      | 62 ± 24         | 581 ± 164       | 1.9            |

*a Each value represents the mean ± SEM from four cultures.

*b Significantly different from KMST-6, with p < 0.05.

*b Significantly different from KMST-6, with p < 0.01.
Studies on the Gene Copy Number of α1(I) and α1(III) Collagen in Transformed Fibroblasts. Because one possible cause of the decrease in the collagen mRNA level in the transformed cell lines might be a reduction in the number of α1(I) and/or α1(III) collagen genes, we analyzed the genomic DNA of the normal and transformed fibroblasts. Genomic DNA fragments of the identical size hybridized to α1(I) collagen, α1(III) collagen, and β-actin probes in all three cell lines with an approximately equivalent intensity (Fig 3). There was no change in the number of α1(I) and α1(III) collagen genes in the transformed fibroblasts. β-actin was used as a standard control to calculate the number of the collagen genes in these experiments.

Transcription of Collagen in Transformed Cells. To determine whether the decreased collagen mRNA at the steady-state level in the transformed cells might be due to a reduced rate of transcription, we performed the run-off assay on nuclei isolated from the normal and transformed fibroblasts (Fig 4). Densitometric values were calculated as the percentage of those of normal fetal fibroblasts (Table III). α1(I) and α1(III) collagen transcription decreased approximately fivefold and tenfold, respectively, in both transformed cell lines, whereas β-actin transcription showed little change in either cell line.

**DISCUSSION**

Malignant transformation of cells induces changes in the rate of synthesis of a number of cellular proteins [1–4]. Many reports have indicated that the rate of synthesis of collagen is decreased in transformed fibroblasts. These studies, however, were done with established or primary cell cultures of animal cells, generally ones derived from mice and chickens [1–3,5–11]. Because any information concerning qualitative and quantitative changes in collagen production in a human system is valuable, we decided to study the collagen synthesis of transformed human fibroblasts. Several reports have indicated that the rate of synthesis of collagen is decreased in SV40-transformed human fibroblasts [12–15]. Using normal diploid

<p>| Table II. m-RNA Levels of Collagen I, Collagen III and β-Actin in Normal and Transformed Fibroblasts |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Cells</th>
<th>Collagen α1(I)</th>
<th>Collagen α1(III)</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMS-6</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>KMST-6</td>
<td>20</td>
<td>8</td>
<td>98</td>
</tr>
<tr>
<td>KMST-6-Ras</td>
<td>16</td>
<td>10</td>
<td>94</td>
</tr>
</tbody>
</table>

* Values are expressed as the percent compared to normal controls (KMS-6). Values given are the average of two independent experiments. All values are normalized for the same amount of RNA.
Figure 3. Southern blot analysis of normal and transformed fibroblasts. Five micrograms of high molecular weight DNA from cells was digested with EcoRI and EcoRI/BamHI endonuclease, electrophoresed on 0.8% agarose gel, and transferred to a nitrocellulose filter. The blot was then hybridized with collagen α1(I), collagen α1(III), and β-actin cDNA probes. N, KMS-6 cells; I, KMST-6 cells; T, KMST-6-Ras cells.

Figure 4. Transcriptional analysis of α1(I) collagen, α1(III) collagen, and β-actin in transformed fibroblasts. Nuclei isolated from normal (KMS-6 cells) and transformed fibroblasts (KMST-6 and KMST-6-Ras cells) were incubated with ATP, UTP, CTP, and [α-32P]-GTP. The 32P-labeled RNA was purified and hybridized to specific DNA immobilized on nitrocellulose filters.

Table III. Results of Densitometry Scanning of Nuclear Run-Off Experiments for Transformed Fibroblasts

<table>
<thead>
<tr>
<th>Cells</th>
<th>Collagen α1(I)* (%)</th>
<th>Collagen α1(III)* (%)</th>
<th>β-Actin* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMS-6</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>KMST-6</td>
<td>18</td>
<td>7</td>
<td>97</td>
</tr>
<tr>
<td>KMST-6-Ras</td>
<td>23</td>
<td>8</td>
<td>91</td>
</tr>
</tbody>
</table>

* Values are expressed as the percent compared to normal controls (KMS-6) and are the average of two independent experiments.

Although the expression of type I and type III collagen genes is regulated in a parallel manner in most cases, differential expression of type I and type III collagen occurs in some cases. For instance, it has been demonstrated that transformed mouse cells in most instances could be a group of type III and type I collagen m-RNA [9]. An unaltered proportion of type I and type III collagen was also observed in SV40-transformed human fibroblasts [14]. On the other hand, under the present experimental conditions, normal fetal human fibroblasts were transformed immortally or neoplastically, the decrease in the m-RNA level and the transcription of type III collagen was two times greater than the normal type I collagen.

The molecular mechanisms responsible for the inhibition of type I and type III collagen RNA transcription in transformed cells are unknown at present. However, according to Liu et al [9] there are at least three possible mechanisms: 1) the presence of a repressor analogous to bacterial or bacteriophage repressors that would act with a specific DNA segment(s) and block the expression of the adjacent gene, 2) a pleiotropic activator of transcription present in normal cells that would become inactive or would not be synthesized in transformed cells, and 3) the occurrence of conformational changes in chromatin around the collagen genes that would prevent the activation of these genes.

Recently, a great deal of information has become available concerning the DNA binding proteins that bind to specific regulatory sequences in the 5' flanking region of type I [32–35] and III [36] collagen genes. There are at least four binding proteins that bind to the regions around −80, −240, −300, and −400 of the α2(I) collagen promoter gene [37]. Among these, the factors that bind to the −80 region and the −300 region of the α2(I) collagen promoter gene [37] have been identified as CCAAT binding factor and nuclear factor 1, respectively [34]. And it has been reported that both of these purified proteins stimulate transcription from several promoters [35,38]. However, unaltered binding activities of these two factors were observed in NIH-3T3 fibroblasts transformed by v-mos or v-ras treated with phorbol myristate acetate, which have previously been shown to produce decreased amounts of type I collagen. Parker et al [32] reported that three distinct retarded bands were observed in the region −360 to +54 of the human α2(I) collagen promoter gene by mobility shift assay with SV40-transformed fibroblast extract, whereas only two of these bands were observed in normal fetal fibroblast extract. This report is very interesting because the absence of α2(I) collagen synthesis in SV40-transformed fibroblast has been shown.

In order to explore the regulation of collagen synthesis in human fibroblasts further, we are attempting to compare the methylation patterns of the collagen genes and the DNA sequence of the promoters region of the collagen gene in normal fetal fibroblasts with those in their transformed counterparts, and are also attempting to

analyze the activity of binding proteins that are known to bind to the collagen promoter genes.

We thank Miss K. Oda for editorial assistance.

REFERENCES

5. Hata R, Peterkofsky B: Specific changes in the collagen phenotype of BALB 3T3 cells as a result of transformation by viruses or a chemical carcinogen. Proc Natl Acad Sci USA 74:2933–2937, 1977