The A204 cell line, derived from a human rhabdomyosarcoma, was studied in culture for its capacity to synthesize collagen types and other extracellular matrix proteins. The cells synthesized and secreted into the culture medium collagenous proteins with apparent molecular weights of 220,000 and 150,000. These were identified as the proa1 and proa2 chains of type V collagen by immunoprecipitation and by peptide mapping. The proa1(V) chain was made in excess of a 2:1 ratio for proa1(V) to proa2(V), and a fraction of the proa1(V) chains together with all of the proa2(V) chains participated in intermolecular disulfide bonding. The chains were extensively glycosylated at hydroxylysyl residues in ascorbate-supplemented cultures. A fraction of the secreted proa1(V) chains was processed to the pal(V) form, but further processing in the culture medium was very slow and the type V collagen molecules deposited in the extracellular matrix apparently retained large non-triple helical domains. Since the A204 cell line does not produce other collagen types, it may prove useful in further studies of the biosynthesis of type V procollagen.

The synthesis of type V collagen has been studied mainly in organ culture. It was shown to be produced by chick embryo neural retina (1), cornea (2, 3), tendon (4), blood vessels (5) and crop (6, 7), and by human placental tissue (8). In cell culture, type V collagen was produced along with other collagen types by fibroblastic (2, 9), endothelial (10, 11), and epithelial cells (12). Considerable interest has focused on this collagen type because of its apparent cell-surface association (13-16) and because it has been found to promote the substrate attachment of certain cell types in the absence of mediator glycoproteins such as fibronectin, laminin, or chondroectin (17, 18).

The structure of type V procollagen has not been clarified despite the fact that the pepsin-resistant domains of this collagen were first described in 1976 (19, 20). Recent biosynthetic studies with chick embryo crop (6, 7), blood vessels (5), and cell cultures (9) have shown that type V procollagen has larger propeptides than interstitial collagen types and retains a considerable part of the nontriple helical segments in the form deposited in tissues. Biosynthetic studies have also confirmed the existence of nondisulfide-linked homotrimer molecules composed of proa1(V) chains (15), as well as disulfide-linked heterotrimers of the form [proa1(V)]2proa2(V) (7, 9). The finding of other disulfide-linked polypeptides, e.g. [proa2(V)]2 (9), suggests that additional combinations of the chains in the triple helix are possible.

To understand thoroughly the structure and processing of type V procollagen, both cell and tissue culture studies on type V collagen biosynthesis are clearly needed. An epithelial cell line from hamster lung has been described that produces a type V procollagen consisting exclusively of a1(V) chains, which is deposited in a pericellular matrix form (15). In this paper, we report on the exclusive synthesis of type V procollagen by an established human rhabdomyosarcoma A204 cell line. The collagen of rhabdomyosarcoma cultures consists of proa1 and proa2 chains that undergo partial disulfide bonding and are mostly found in the culture medium, where they undergo limited processing. In addition to its utility in biosynthetic studies, this cell line may prove useful for the preparation of type V procollagen as an immunogen and as a source of human type V collagen-specific cDNA for cDNA clones and for screening genomic libraries.

**MATERIALS AND METHODS**

**Cell Culture and Metabolic Labeling**—The A204 cells were obtained from Drs. Joseph E. DeLarco and George J. Todaro (Laboratory of Viral Carcinogenesis, National Cancer Institutes, Bethesda, MD). The cells were grown in Eagle’s Minimal Essential Medium or Dulbecco’s modified medium, supplemented with 10% fetal calf serum, 100 units of penicillin/ml, and 50 μg of streptomycin/ml. Cultures were split twice a week at a 1:2 to 1:8 ratio. Subconfluent to confluent cultures were labeled, usually for 21 to 24 h, with 20 to 50 μCi/ml of [2, 3-3H]proline (35 Ci/mmol, New England Nuclear), or with [3H]proline in combination with [2-14C]glycine (50 μCi/ml, 23 Ci/mmol, Amersham/Searle Co.) in DMEM containing 50 μg/ml of sodium ascorbate and 50 μg/ml of β-aminopropionitrile formate. For experiments in which radioactive 3-hydroxyproline was to be determined, 20 μCi/ml of L-(5-3H)proline (29 Ci/mmol, Amersham/Searle Co.) was used. For determination of hydroxylysine and glycosylated hydroxylysine, labeling was performed with L-[U-14C]lysine (50 μCi/ml, New England Nuclear).

**Preliminary Fractionation of Culture Medium Protein**—Immediately after removal of labeled culture medium from the cells, protase inhibitors were added to a final concentration of 0.2 mM PMSF, 10 mM MaLN&El, and 2.5 mM EDTA, pH 7.5. The cell layers were washed once at 4 °C with phosphate-buffered saline containing protease inhibitors and the wash was combined with the medium. The sample was clarified by centrifugation and subsequently chilled to 0 °C. For screening and initial characterization, small aliquots of culture medium protein were precipitated in 10% trichloroacetic acid containing 18 U.S.C. Section 1734 solely to indicate this fact.

* This work was supported in part by National Institutes of Health Grants HL18645, AM11248, and DE02600, and by a grant from R. J. Reynolds Industries, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18

**The abbreviations used are:** DMEM, Dulbecco’s modification of Eagle’s medium; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; MaLN&El, N-ethylmaleimide; NaDodSO4, sodium dodecyl sulfate.

**Received for publication, January 11, 1982**

**Biosynthesis of Type V Procollagen by A204, a Human Rhabdomyosarcoma Cell Line***

Kari Alitalo, Raili Myllylä, Helene Sage, Pam Pritzl, Antti Vaheri, and Paul Bornstein

From the Department of Biochemistry, University of Washington, Seattle, Washington 98195, and the Department of Medical Biochemistry, University of Oulu, 90220 Oulu 22, Finland, and the *Department of Virology, University of Helsinki, 00290 Helsinki 29, Finland

*This was supported in part by National Institutes of Health Grants HL18645, AM11248, and DE02600, and by a grant from R. J. Reynolds Industries, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Fellow of the National Institutes of Health Fogarty International Center (F05 TW 03138).

‖ An Established Investigator of the American Heart Association.
biosynthesis of type V procollagen

ing 50 μg/ml of BSA and 1 μg/ml of peptatin A (Protein Research Foundation, Osaka, Japan). The remaining precipitate was dissolved in a 1:1000 solution of 1 M Tris-Cl, pH 7.0, and the resulting precipitate was removed by centrifugation for 30 min at 48,000 × g. Ammonium sulfate was then added to the supernatant solution to a final concentration of 50% (w/v), and the precipitate was recovered by centrifugation. The 50% ammonium sulfate precipitate was dialyzed against 0.1 M acetic acid containing 0.5 μg/ml of peptatin A and was lyophilized.

Culture medium proteins were resolved by DEAE-cellulose (DE-52, Whatman) chromatography; proteins were bound in 4 mM urea, 75 mM NaCl, 50 mM Tris-HCl, pH 7.5, containing 2.5 mM EDTA and 0.2 mM PMSF, followed by elution with a linear salt gradient from 75 to 375 mM NaCl (conductivity = 12 mmho) and finally with 1 M NaCl (6).

\[
\text{NadDSoS-Polyacrylamide Gel Electrophoresis and Fluorescence Autoradiography-} \text{Proteins were resolved on discontinuous polyacrylamide slab gels containing 0.5 M urea (21), as described by Crouch and Borchstein (22). The bands were visualized either after staining with Coomassie Brilliant Blue R or after processing for fluorescence autoradiography (23, 24). A scanning densitometer was used to measure protein-containing bands in the linear absorbance range.}
\]

\[
\text{Bacterial Collagenase Digestion-Samples were dissolved in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl}_2, \text{0.5% Triton X-100, 0.2 mM PMSF, and 10 mM MalNEd, and spun briefly at 15,000 × g for 5 min at a microfuge in a buffer (Microgen). Bacterial collagenase (50 μl) (Advance Biofluaxes, form II), which was dissolved in 0.33 M glycerol, 0.025 M Tris-HCl, pH 7.4, at a final concentration of 100 μg/ml, was added to each sample; controls received 50 μl of buffer. The samples were incubated at 37 °C for 2 h, after which they were chilled to 0 °C and precipitated in 10% trichloroacetic acid. To the pellets were added 5 μl of 1 NaOH plus 45 μl of NadDSoS-Polyacrylamide gel electrophoresis sample buffer containing 50 mM dithiothreitol (22), and aliquots were measured by liquid scintillation counting prior to analysis by NadDSoS-polyacrylamide gel electrophoresis.}
\]

\[
\text{Isolation of Cell Layer Collagen-Cell layers were scraped with a rubber policeman into 0.5 M acetic acid containing 0.5 μg/ml of peptatin A at 0 °C and dialyzed versus 0.5 M acetic acid. The dialysate was then centrifuged at 48,000 × g for 30 min, and the pellet and supernate were lyophilized separately.}
\]

\[
\text{The lyophilized protein was suspended at 1 mg/ml in 0.5 M acetic acid at 4 °C. Pepsin ( Worthington, twice recrystallized, dissolved in water at 10 mg/ml) was added at an enzyme to substrate weight ratio of 1:50, and the digestion proceeded for 23 h at 4 °C. The digest was clarified by centrifugation at 48,000 × g for 30 min, and collagenous protein was precipitated by addition of NaCl to 1.8 M, accompanied by precipitation of proteins. Alternation of the digest was performed with 8 M NaOH for 30 min, after which the pH was lowered to 2.2 and the proteins were precipitated at a concentration of 1.8 M NaCl by stirring overnight.}
\]

\[
\text{The precipitates containing cell layer collagen were isolated by cesium chloride gradients in a Beckman ultracentrifuge} \text{dissolved in and dialyzed against 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5, containing 0.2 M PMSF. Insoluble material was removed by centrifugation, dissolved in and dialyzed against 0.1 M acetic acid, and lyophilized. Protein which was soluble in 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5, was precipitated at a concentration of 4.5 M NaCl, subsequently dissolved in and dialyzed against 0.1 M acetic acid, and lyophilized.}
\]

\[
\text{Comparative Peptide Mapping-} \text{Pepsin-digested cell layer collagen was cleaved with CNBr (Pierce) according to previously described methods and was thus able to interchange reversibly. As originally described by Cleveland et al. (26) and further modified for single column microanalysis.}
\]

\[
\text{Cell Culture-The A204 cell line was originally established from a human rhabdomyosarcoma by Giard et al. (33). These cells consist of two morphologic phenotypes that are apparently able to interchange reversibly. As originally described by Giard et al. (33), the cell line had a bimodally distributed chromosome number with diploid and tetraploid cells present. The cells grow as a substrate-attached oligolayer and can be maintained for over 3 days in serum-free medium without apparent loss of cell viability. We used cells subcultured 3} \text{to 5 times with essentially similar results. Our preliminary study}
\]
(34) had indicated that these cells produced unusual collag-

enous polypeptides. The present experiments were under-
taken to identify and characterize the procollagens of A204
cells.

**Identification of Collagenous Proteins**—The cells, grown

on plastic dishes, were labeled with tritiated proline and
proteins in the culture media were analyzed by NaDodSO4-
polyacrylamide gel electrophoresis under nonreducing and
reducing conditions. In gels stained for protein, we identified
three major nondisulfide-linked polypeptides with apparent
molecular weights of 220,000, 190,000, and 130,000 (Fig. 1,
Lane 1). In addition, there were minor polypeptides with $M_r$
= 290,000 under nonreducing conditions and with $M_r$ = 150,000
under reducing conditions (Lanes 1 and 2). Comparison of the
gels by autoradiography and protein staining showed that the
above polypeptides were labeled at roughly equal specific
radioactivities, both with proline (Fig. 1, Lane 3) and with
glycine (not shown). The polypeptides with molecular weights
of 220,000, 190,000, and 150,000 (as well as the $M_r = 290,000$
band), together with some minor radiolabeled polypeptides,
were susceptible to digestion with bacterial collagenase (com-
pare Lanes 3 and 4). In the cell layers, only two polypeptides
were digested by collagenase. These had mobilities corre-
sponding to the 220,000 and 150,000 species found in the
culture media (Lanes 5 to 8).

Pepsin digestion of the proteins in A204 cell layers, followed

by salt fractionation and gel electrophoresis, produced two
collagenase-sensitive nondisulfide-bonded polypeptide bands
with mobilities of type V collagen $\alpha_1$ and $\alpha_2$ chains (Fig. 2A).

Pepsin-resistant collagen from culture media had an excess of
$\alpha_1$(V)-sized chains compared to $\alpha_2$(V)-sized chains and, in
addition, contained minor amounts of a collagenase-sensitive
polypeptide migrating between $\alpha_1$(V) and $\alpha_2$(V) (Fig. 2B,
Lanes 5 and 6). The latter was not studied further. Pepsin-
resistant material from the cell layers did not usually contain
a third chain.

**Ion Exchange Chromatography and Peptide Mapping**—
The procollagens secreted by A204 cells bound to DEAE-
cellulose and were eluted only at relatively high salt concen-
trations (corresponding to conductivities greater than 6.5
mmho; Fig. 3A). Gel electrophoresis of the procollagen-con-
aining fractions under reducing and nonreducing conditions
showed that a fraction of the 220,000- and 150,000-dalton
polypeptides was disulfide-linked into higher molecular
weight forms (Fig. 3B, compare the analysis of fraction III in
the presence and absence of dithiothreitol).

The procollagen polypeptide bands with molecular weights
of 220,000 and 190,000 were cut out of stained gels, iodinated
at primary amino groups, digested with proteinase K and
subjected to two-dimensional analysis by electrophoresis and
chromatography (27). The results are shown in Fig. 4. It can
be seen that the two procollagen polypeptides (A and B)
resemble each other, suggesting that they are derived from a
common chain. Both polypeptides show a greater resemblance
to $\alpha_1$(V) obtained from pepsin-treated placental type V col-
lagen than to the corresponding placental $\alpha_2$(V) chain (27).
The differences between the cell culture and placental chains
may be attributed to the pepsin-sensitive propeptides in the
rhabdomyosarcoma cell procollagen and to differences in post-
translational modification of lysine residues (see below).

Evidence for the existence of the pro$a_2$(V) chain was pro-
vided by proteolytic mapping of the pepsin-resistant polypep-
tides in the culture medium of A204 cells (Fig. 5). Comparison
of one-dimensional peptide maps of standard $\alpha_1$(V) and $\alpha_2$(V)
chains with maps of A204 pepsin-resistant chains revealed
characteristic $\alpha_2$ chain-specific fragments in the latter pattern
(Fig. 5, arrows). This identification was substantiated by
CNBr cleavage of $\alpha_2$(V) chains from culture medium (data
not shown). We therefore conclude that A204 cells synthesize
both the pro$a_1$(V) and pro$a_2$(V) chains.

**Immunoprecipitation and Immunofluorescence**—Double
antibody immunoprecipitation with anti-type V collagen IgG
precipitated maximally about 9% of total acid-insoluble [3H]
proline-labeled protein in the culture medium of A204 cells
(Fig. 6A), while only negligible amounts of radioactivity were
present in precipitates obtained using anti-type I or anti-type
III procollagen or with anti-type IV or anti-intimal (30) col-
lagen IgG (Table I). Analysis of immunoprecipitates by gel
electrophoresis demonstrated the presence of pro$a_1$(V) chains
in the precipitate generated with anti-type V serum; specific
precipitation of other collagen types from human fibroblast
culture medium was observed using other antisera (Fig. 6B).
FIG. 2. Polyacrylamide gel electrophoresis and autoradiography of [3H]proline-labeled pepsin-resistant proteins in A204 cell layers (A) and culture media (B) as analyzed under non-reducing (-DTT) and reducing (+DTT) conditions. A, Lanes 1 and 4, starting material; Lanes 2 and 5, pepsin-resistant material insoluble in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5; and Lanes 3 and 6, pepsin-resistant material soluble in the same buffer. Lane 7 shows type I procollagen standard. The positions of migration of placental type V collagen α1 and α2 chains are indicated. Proteins were analyzed by autoradiography in a composite 6%/10% polyacrylamide gel. B, proteins secreted by A204 cells during a 24-h period were fractionated by ammonium sulfate precipitation. Lane 1, material soluble in 50% (NH₄)₂SO₄; Lane 2, material precipitated by 30 to 50% (NH₄)₂SO₄; Lane 3, material precipitated by 30% (NH₄)₂SO₄. Lanes 4 to 6, Lanes 1 to 3, respectively, treated with pepsin. The 220,000 and 190,000 collagenous polypeptides as well as the mobility of placental collagen α1(V) and α2(V) chains are indicated. Proteins were resolved on a 5% polyacrylamide gel and were visualized by staining with Coomassie blue. The arrow indicates the top of the 5% gel. DTT, dithiothreitol.

No binding to A204 cell layers was found in double immunofluorescence analysis with any of the IgGs except anti-type V collagen. Type V collagen antigenicity was localized to fibrillar structures surrounding individual A204 cells (Fig. 7, A and B) and was also demonstrated intracellularly when staining was performed after detergent extraction of fixed cell layers (not shown). Analyses for fibronectin and laminin using the above techniques gave consistently negative results.

Two cell types with distinct types of intermediate filaments are found in rhabdomyosarcomas (35). One cell type stains only for desmin which is specific for muscle cells, while the other cell type is positive for vimentin (35). The A204 cells were uniformly vimentin-positive (Fig. 7, C and D); this property could represent an in vitro selection of particular cells of the original tumor.

Synthesis and Secretion of proα1(V) and proα2(V) Chains—Type V procollagen is secreted as partially disulfide-linked heterotrimer and non-disulfide-linked homotrimer molecules from chick embryo tendon fibroblasts (9). Since A204 cells produced only type V collagen, it was possible to follow

FIG. 3. DEAE-cellulose chromatography of [3H]proline-labeled culture medium protein from A204 cells (A) and analysis of fractions by gel electrophoresis (B). Arrows indicate the start of the NaCl elution gradient and the final elution with 1 M NaCl. Fractions were pooled as indicated by Roman numerals. Approximately 1.5 x 10^6 cpm from each pool was analyzed by 5% NaDodSO₄-polyacrylamide gel electrophoresis both in the presence and absence of 50 mM dithiothreitol (DTT) (B). SM, starting material; Roman numerals refer to the fractions indicated in A; numbers indicate the mobility of collagenous polypeptides and their estimated molecular weights in thousands. (Note disulfide linking of the 220,000 and 150,000 molecular weight collagenous components in fractions III and IV).
Fig. 4. Proteinase K peptide maps. A, $M_r = 220,000$ polypeptide; B, $M_r = 190,000$ polypeptide.

Fig. 5. Proteolytic mapping of pepsin-treated collagen of A204 culture medium by staphylococcal V8 protease. Ten micrograms of placental collagen $\alpha_1(V)$ chains, 20 $\mu$g of $\alpha_2(V)$ chains or 10 $\mu$g of placental collagen containing both $\alpha_1(V)$ and $\alpha_2(V)$ chains together with radiolabeled A204 collagen (200,000 cpm. Lane A204) were digested with 5 $\mu$g of V8 protease for 1 h at room temperature. Polypeptides were analyzed by electrophoresis in a 10% polyacrylamide gel. The gel was stained for protein and was subsequently processed for fluorescence autoradiography (Lane A204). The analysis of intact starting material is also shown. Arrows show polypeptides specific for cleaved $\alpha_2(V)$ chains. The polypeptides of the staphylococcal enzyme are marked with brackets.

Fig. 6. Immune titration of labeled type V collagen using double antibody precipitation (A) and polyacrylamide gel analysis of collagen immunoprecipitates from culture media of A204 cells and human fetal fibroblasts (HFF) (B). Experiments were performed as described under "Materials and Methods"; starting material contained $3 \times 10^7$ cpm. B, Lane 1, A204 medium + anti-type V collagen; Lane 2, A204 medium protein precipitated in 10% trichloroacetic acid; Lane 3, human fetal fibroblast medium + anti-type III collagen; Lane 4, human fetal fibroblast medium + anti-type I collagen; Lane 5, human fetal fibroblast medium protein, precipitated in 10% trichloroacetic acid. Processing of type I procollagen appeared to have occurred in the immunoprecipitate (compare Lanes 4 and 5).

The intracellular fate of newly synthesized type V procollagen chains. Pulse-chase experiments (Fig. 8) showed disulfide bond formation among proα2(V) chains and a fraction of proα1(V) chains. Upon secretion (after a chase time of about 80 min), part of the proα1(V) chains was cleaved to the faster migrating (190,000-dalton) form [proα1(V)]. However, in rhabdomyosarcoma cultures there was very little processing after this step. Even after a chase period of 3 days, the pattern of polypeptides in the culture medium was relatively unchanged (not shown). This observation was consistent with the slow accumulation of further processed (faster migrating) chains in the cell layer. Polypeptides from the A204 cell layers did not contain α-chain-sized type V collagen chains.

We did not undertake studies of the chain assembly and
composition of type V collagen in A204 cell cultures. Disulfide linking and peptide maps of the 290,000 polypeptide band suggest that it represents heterodimers of the form, proα1(V)-proα2(V), and disulfide-linked trimeric procollagens would have remained on top of the separating 5% polyacrylamide gels which were used in these experiments. In fact, the presence of such trimers can be inferred from the pulse-chase experiment shown in Fig. 8 (lanes at a 40-min chase and thereafter).

Post-translational Modifications—Measurements of post-translational modifications in the collagen synthesized by A204 cells are presented in Table II. The degree of hydroxylation of proline was low (about 30%) in the procollagen chains but about 50% of proline residues were hydroxylated in the pepsin-resistant chains. The higher percentage probably is due to the removal of noncollagenous propeptides from the precursor chains. The 3-Hyp/4-Hyp ratio was somewhat variable (0.032 to 0.016) but consistent with the identity of the rhabdomyosarcoma collagen as distinct from type IV collagen.

Table I

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Radioactivity precipitated (cpm (%)</th>
<th>A204 cultures</th>
<th>Human fetal fibroblast</th>
<th>Cell layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td>145,904 (100)</td>
<td>124,157 (100)</td>
<td></td>
</tr>
<tr>
<td>Anti-procollagen type I</td>
<td></td>
<td>422 (&lt;1)</td>
<td>55,104 (44)</td>
<td></td>
</tr>
<tr>
<td>Anti-procollagen type III</td>
<td></td>
<td>576 (&lt;1)</td>
<td>6,124 (4)</td>
<td></td>
</tr>
<tr>
<td>Anti-collagen type IV</td>
<td></td>
<td>552 (&lt;1)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Anti-intimal collagen</td>
<td></td>
<td>492 (&lt;1)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Anti-collagen type V</td>
<td></td>
<td>8,208 (6)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Preimmune rabbit serum</td>
<td></td>
<td>192 (&lt;1)</td>
<td>504 (&lt;1)</td>
<td></td>
</tr>
</tbody>
</table>

* n.d., not determined.

Table II

<table>
<thead>
<tr>
<th>Chain</th>
<th>4-Hyp/4-Hyp</th>
<th>3-Hyp/4-Hyp</th>
<th>Hydroxylation of Prolyline</th>
<th>Glycosylation of Hydroxylysine</th>
<th>Glucosylation of Glycosylated Hydroxylysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A204 cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proα1(V)</td>
<td>0.37</td>
<td>2.4</td>
<td>33</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td>Proα2(V)</td>
<td>0.39</td>
<td>2.3</td>
<td>46</td>
<td>90</td>
<td>97</td>
</tr>
<tr>
<td>Pepsin-resistant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a1(V)</td>
<td>0.47</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell layer</td>
<td>0.52</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pepsin-resistant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a2(V)</td>
<td>0.49</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissues</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a1(I)</td>
<td>0.48</td>
<td>2.8</td>
<td>64</td>
<td>97</td>
<td>85</td>
</tr>
<tr>
<td>a2(I)</td>
<td>0.52</td>
<td>2.8</td>
<td>57</td>
<td>33</td>
<td>63</td>
</tr>
<tr>
<td>a2(II)</td>
<td>0.43</td>
<td>1.0</td>
<td>14</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>IV†</td>
<td>0.57</td>
<td>7.2</td>
<td>82</td>
<td>76</td>
<td>94</td>
</tr>
</tbody>
</table>

* Values obtained from a recent review (36).
† Values pertain to type IV procollagen from cultured human amniotic fluid cells (37).

Fig. 7. Indirect immunofluorescence analysis for extracellular matrix and vimentin filaments in A204 cultures. Cell layers were stained after fixation with p-formaldehyde, treated with antiserum (1:40 dilution) and then with fluorescein-conjugated sheep anti-rabbit IgG. A, phase contrast; B, anti-type V collagen fluorescence in the field shown in A; C, anti-vimentin staining of p-formaldehyde-fixed, detergent-permeabilized cells; D, phase contrast of C. Magnifications: × 800.
Comparison with data on pepsin-digested type V collagen from tissues indicated a relatively high degree of lysyl hydroxylation and hydroxylysyl glycosylation in ascorbate-supplemented A204 cell cultures. Comparison of the degree of hydroxylation and glycosylation of collagen in A204 cell cultures with that in other tumor cell lines has been the subject of another report (38).

Secretion of type V procollagen by A204 cell cultures was inhibited by 0.3 mM $\alpha\alpha'$-dipyridyl, a chelator of the ferrous ion cofactor required for peptide hydroxylation (data not shown). The intracellular unhydroxylated proα1(V) proα2(V) chains and their disulfide-linked forms. The evidence is based on identification of the procollagen by collagenase digestion and gel electrophoresis, the characteristic elution from DEAE-cellulose at relatively high salt concentration, peptide mapping, and immunoprecipitation. Also, pepsin treatment of both the culture medium and of cell layers resulted in α1(V)- and α2(V)-sized collagen chains whose identity was further established by peptide mapping. Rhabdomyosarcoma collagen showed a high degree of both prolyl and lysyl hydroxylation and a high degree of hydroxylysyl glycosylation in ascorbate-supplemented culture conditions.

On the basis of analyses of tissue collagens, conflicting findings regarding the structure of type V collagen have been reported. The structure ($\alpha\alpha\alpha$)αββ was first proposed by Burgeson et al. (19) for fetal membrane type V collagen. However, subsequent studies (25, 39, 40) were not compatible with a 2:1 ratio of α1(V) to α2(V), and further studies led to the suggestion that two different collagen molecules with compositions ($\alpha\alpha\alpha$)αββ and ($\alpha\alpha\alpha$)βββ existed (41, 42). On the other hand, the physiochemical measurements of Bentz et al. (43) clearly showed the presence of two different chains in type V collagen molecules from amniotic membranes. A third distinct chain, α3(V), was discovered by Brown et al. (40) and characterized by Sage and Bornstein (25). In addition, other, possibly related, chains have been reported in cartilage (44, 45).

Biosynthesis of type V collagen accounted for only about 5% of total collagen synthesis by secondary cultures of chick embryo tendon fibroblasts (9). The studies of Haralson et al. (15) showed that Chinese hamster lung cell cultures produced exclusively molecules composed of α1(V) chains, and disulfide-linked heterodimers and trimers have been found in chick embryo crop tissue (7).

Type V procollagen was secreted from A204 cells as mole-

---

**Fig. 8.** Pulse-chase experiment with A204 cultures and analysis by polyacrylamide gel electrophoresis. Cells were labeled for 15 min with [3H]glycine and [3H]proline and were then incubated with unlabeled amino acids for the indicated times in minutes. Cell aliquots were analyzed by NaDodSO₄-gel electrophoresis on 5% gels for 15 min with [3H]glycine and [3H]proline and were then incubated under nonreducing and reducing conditions. Arrows show the fluorography imprints of proα1(V), proα2(V), and proα3(V) chains and their disulfide-linked forms. DTT, dithiothreitol.

**Fig. 9.** Change in the mobility of intracellular proα1(V) and proα2(V) chains in response to treatment with $\alpha\alpha'$-dipyridyl, as analyzed by NaDodSO₄-polyacrylamide gel electrophoresis.
cules containing proα1(V) and proα2(V) chains. The former were present in excess of 2:1 over the latter. This finding is unlike that in the hamster epithelial cell cultures described by Haraldson et al. (15), in which proα2(V) chains were not detected. In A204 cells, proα2(V) chains and a fraction of proα1(V) were disulfide-linked to form high molecular weight oligo/multimers, but we did not analyze the composition of these complexes. It is likely that they correspond to the dimers and trimers found by others (5-7, 9). Intrachain disulfide bonds in the nontriple helical extensions (propeptides) may account for the slightly lower mobility, after reduction, of proα1(V) and proα1(V) chains during electrophoresis; this apparent decrease was not observed for the α1(V) chains. We have shown, in unpublished experiments, that after a 5-min pulse disulfide-linked forms are first detected in cells after a 30-min chase period. The secretion time, as estimated from the pulse-chase experiments, is approximately 100 min, a value similar to the secretion time of type IV procollagen and longer than that of interstitial collagens.

Chelation of Fe³⁺ ions by 0.3 mM α,α'-dipyridyl completely prevented the appearance of type V procollagen in the culture medium. At the same time, the procollagen chains recovered from the cell layer were altered in electrophoretic mobility, probably due to lack of hydroxylysyl glycosylation. However, such procollagen chains (which may still contain N-glycosidically linked oligosaccharide side chains) exhibited electrophoretic mobilities on polyacrylamide gels corresponding to Mₐ = 200,000 for the proα1(V) chains and Mₐ = 145,000 for the proα2(V) chain. Thus, the large apparent size of these chains is not due solely to a high degree of side chain modification.

Biosynthesis and secretion of type V collagen is maintained in A204 cell cultures for over 60 passages and thus seems to be a constitutive feature of this rhabdomyosarcoma cell line. Earlier, we studied another well known rhabdomyosarcoma cell line, RD, and found the production of fibronectin and types III, IV, and V procollagens by these cells (46). All of these collagen types are found in the endomysium of skeletal muscle (46-48) and changes in the collagen types have been reported during development of chick muscle tissue (48). It is thus possible that the different results observed with the two rhabdomyosarcoma cells are due to different degrees of differentiation of these cell lines. This conclusion is consistent with the observations of Sasse et al. (49) regarding the origin of collagen types in avian myogenic cultures. Sasse and collaborators (49) found mononucleated cells in myogenic clones that produced types I and II collagens, while myotubes were positive only for type V collagen as determined by immunofluorescence. Differentiating avian myoblasts also cease fibronectin production (50). In view of the recent advances in culturing human skeletal muscle cells (51), it should be possible to understand the regulation of matrix protein biosynthesis during the differentiation of these cells.

For studies of procollagen types, tumor cell lines provide a convenient source of soluble material that is often processed more slowly than in the corresponding normal cell cultures (34). Although only approximately 10% of labeled peptidyl proline radioactivity was in collagen in the A204 culture medium, sufficient protein to be visible by Coomassie blue staining of a gel was secreted by 10⁷ cells in 24 h in the presence of 50 μg/ml sodium ascorbate. Both processing and degradation of the procollagen was slow, allowing for large-scale preparation. Furthermore, since labeled protein in A204 cell layers can be solubilized in detergents, intracellular modifications and secretion as well as accumulation of underhydroxylated chains can be followed. Earlier studies have used tissue extracts (6, 7) and pepsin digestion (15) for the same purpose. Although α-sized collagenous polypeptides were not seen in the cell layers, even after long labeling times, polypeptides about a third larger were found in the pericellular matrix. These may correspond to the forms that still retain large, pepsin-sensitive telopeptides, as described by Kumamoto and Fessler (6, 7).

The A204 cell line is an exception to the observation that the majority of type V collagen produced in culture is confined to the cell layer (see Ref. 52 for a review). Indeed, it has been suggested that this collagen functions as a cell surface-associated protein (15, 16). Furthermore, type V collagen may have a special role on the surface of some cell types. Thus, it has been shown that smooth muscle cells produce type V collagen (53) and that attachment to this collagen is mediated by a trypsin-resistant cell surface glycoconjugate (18). The cell surface binding site on type V collagen appears to be located in the α2(V) chain (18). Therefore, the chain composition of type V collagen, which may vary in different tissues, may be of physiological significance.

Our immunofluorescence results help to establish the distribution of type V collagen in the pericellular matrix. Rabbit IgG prepared against pepsin-solubilized human placental [α1(V)2α2(V) collagen stained extracellular matrix fibrils in a manner similar to the fibrils composed of interstitial collagen in fibroblast cultures (54, 55). Indeed, the cells often exhibited a close association with the fibrils along their surface, but no indication of staining of the plasma membrane, independent of the extracellular matrix, was obtained. It remains possible that different molecular forms of type V collagen have a different distribution.

We plan to use A204 cells for production of an immunogen that can generate type V collagen propeptide-specific monoclonal antibodies. These antibodies will be used to clarify the degree of processing of this collagen type that takes place in tissues. The A204 cell line should also prove useful for isolation of proα1(V) and proα2(V) chain messenger RNAs and, eventually, for cloning of type V collagen-specific cDNAs.

Acknowledgments—We thank Drs. Françoise and Giulio Gabbiani for the immunofluorescence staining with anti-vimentin antibodies and Clarice Martin for preparation of the manuscript.

REFERENCES


Biosynthesis of Type V Procollagen

42. Rhodes, R. K., and Miller, E. J. (1978) Biochemistry 17, 3442-3448
52. Myllylä, R., Alitalo, K., and Vaheri, A. (1978) Biochemistry 17, 446-452