We previously showed that soluble, pepsin-solubilized collagen VI increases de novo DNA synthesis in serum-starved HT1080 and Balb 3T3 fibroblasts up to 100-fold compared with soluble collagen I, reaching 80% of the stimulation caused by 10% fetal calf serum. Here we show that collagen VI also inhibits apoptotic cell death in serum-starved cells as evidenced by morphological criteria, DNA laddering, complementary apoptosis assays (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, enzyme-linked immunosorbent assay, and fluorescence-activated cell sorting analysis), whereas cells exposed to medium and collagen I where arrested at G1-S. This was accompanied by a 2–3-fold increase in cyclin A, B, and D1 protein expression. Collagen VI-induced inhibition of apoptotic cell death may be operative during embryogenesis, wound healing, and fibrosis when elevated tissue and blood levels of collagen VI are observed, thus initiating a feedback loop of mesenchymal cell activation and proliferation.

Apoptosis or programmed cell death is a distinct form of active self-destruction. It is an important mechanism by which cells can be eliminated from the organism, e.g. during tissue remodeling, embryogenesis, and immune elimination. Apoptosis is characterized by typical morphological changes and triggered by biochemically defined events (1). Morphologically, apoptotic cells are characterized by a reduction in cell size, chromatin condensation, and membrane blebbing. DNA fragmentation into multiples of 180 base pairs is regarded as the hallmark of apoptosis, but degradation into larger DNA fragments has also been described (2). Intracellular effectors of apoptosis are the caspases, enzymes that degrade key structural and functional molecules of the cell, such as nuclear lamins, cytoskeletal proteins, protein kinases, and DNA repair enzymes (3). The Bcl-2 family of proteins plays an important role in the regulation of apoptosis upstream of caspases. Their antiapoptotic members close and their proapoptotic members open mitochondrial membrane pores and thus regulate the release of cytochrome C into the cytosol. Cytochrome c can then activate procaspase 9 via formation of a complex with Apaf-1 (4). Bcl-2 family proteins function as homo- and heterodimers with the ratio of death promoters to death suppressors presumably determining if a cell enters the apoptotic pathway (5).

Apoptosis depends on signaling processes that derive from extracellular or intracellular events. The tumor suppressor gene p53, regarded as “guardian of the genome,” is an example of intracellular regulation, because it is up-regulated in response to DNA damage and, in turn, can slow cell cycle progression and/or promote apoptosis by inducing the expression of the Bcl-2 family member Bax (6). Extracellularly, death factors such as tumor necrosis factor and Fas ligand, by binding to their receptors, trigger a caspase cascade via adaptor proteins (7). Other extracellular factors (e.g. interleukin-2 and -3 as well as nerve growth factor and insulin-like growth factor-1) provide survival rather than death signals for specialized cells (8).

Whereas much is known about regulation of apoptosis by growth factors, cytokines, and hormones, the role of the extracellular matrix (ECM)1 in regulating apoptosis, although well recognized, is yet little understood (9, 10). The most common approach to study the effect of ECM on apoptosis is to plate cells on precoated ECM components (11). Using this approach, many investigators have demonstrated that integrin-mediated adhesion and spreading on ECM molecules is necessary for the survival of many cell lines. Adhesion-dependent cells that are deprived of anchorage die by an apoptotic mechanism called anoikis (12, 13). The basement membrane has been shown to prevent apoptosis through β1-integrin signaling (14), and several distinct ECM molecules such as fibronectin or collagens have been found to facilitate survival through integrin-mediated signal transduction pathways (15).

Collagen VI (CVI) is a large, multidomain ECM protein composed of a triple-helix of the chains α1, α2, and α3, which, via tetramerization and end-to-end association, assemble into a microfibrillar network in vivo and in vitro (16). Its pattern of distribution and its unique structure and expression compared with other ECM molecules indicate that CVI might fulfill spe...
cialized tasks in tissue organization and cell functioning (17–19). In this line, CVI interacts with various other matrix components including hyaluronic acid (20), syndecan (21), decorin (22), von Willebrand factor (23), and collagens, types I, IV and XIV (19, 24). CVI has been shown to be highly expressed among mesenchymal cells during development of liver (17) and eyes (25), suggesting a role in cellular differentiation and survival (26). In this line, strong expression of CVI has been reported among malignant tumors like melanoma and glioma, which show more invasive behavior than those not expressing CVI (27, 28). Native, triple-helical CVI as well as its single chains promote cell attachment, spreading, and proliferation through at least partially integrin-independent mechanisms (29, 30).

Here we describe the unique effect of soluble CVI, in the absence of growth factors, to promote cell survival through down-regulation of the proapoptotic Bax protein. Furthermore, CVI sets the stage for cell cycle progression by up-regulating cyclins A, B, and D1.

**EXPERIMENTAL PROCEDURES**

If not stated otherwise, all reagents were obtained from Sigma and were of the highest purity grade available.

**Collagen Isolation**—Human collagen I (CI) and CVI were isolated from human placenta by pepsin digestion, fractional salt precipitation in acidic and neutral buffers, ion exchange, and molecular sieve chromatography as described previously (30). All preparations were tested for purity by SDS-PAGE and amino acid analysis after hydrolysis in 6 M HCl under nitrogen for 24 h at 110 °C. Purified collagens were lyophilized and redissolved in 0.15 or 0.5 M acetic acid before use. Growth factor contamination of CVI was excluded by pepsin digestion, as described previously (30).

**Cell Culture**—Balb/3T3 cells (mouse fibroblasts, ATCC CCL-163) and HT1080 cells (human fibrosarcoma cells, ATCC CCL-121) were cultured in 80-cm² flasks using Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany) supplemented with penicillin (100 mg/liter), streptomycin (10 µg/ml), acetic acid (50 µg/ml), and 10% fetal calf serum (FCS) under conditions of 5% CO² and 100% humidity at 37 °C and a 95% oxygen atmosphere. Cells were then serum-starved for 24 h, with no FCS (starving medium), or 10% FCS (FCS group)). A defined positive control was prepared using a lysozyme control (lysis buffer alone) divided by the OD of the corresponding control (viable cells). The isolated DNA in each group was sheared with an insulin needle before centrifugation at 13,000 rpm for 20 min to separate low molecular weight DNA (oligonucleosome-sized fragments derived from apoptotic cells) from high molecular weight DNA (from viable cells). 400 µl of the supernatant containing oligonucleosomes were carefully transferred to another Eppendorf tube and diluted 1:5 with lysis buffer. A 100-µl aliquot was used for the ELISA, which was performed according to the manufacturer’s instructions (Cell Death Detection ELISA; Roche Molecular Biochemicals). In brief, antibodies against histones were coated onto 96-well, 100-µl lysis samples containing oligonucleotides, and histones were added and incubated for 1 h. After washing, the wells were incubated with anti-double strand DNA antibody coupled to peroxidase for 1 h. After reaction with peroxidase substrate, absorbance was measured at 405 nm using an ELISA reader. Background values (lysis buffer alone) were subtracted, and results are shown as enrichment factor (enrichment factor = OD (optical density) of the sample (apoptotic cells) divided by the OD of the corresponding control (viable cells)).

**DNA Laddering**—After a 24-h treatment with medium, CI, CVI, or 10% FCS, cells were trypsinized and adjusted to equal cell number (1.5 × 10⁶). The Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals) was used according to the manufacturer’s instructions, leading to isolation of oligonucleosomes. The isolated DNA in each group was electrophoresed on a 1.5% agarose gel at 75 V for 1.5 h, stained with ethidium bromide, and visualized via a UV light source (302 nm). As a positive control, apoptotic DNA from HL60 cells (promyelocytic leukemia) was used.

**Cell Death Detection ELISA**—4 × 10⁶ cells were plated in six-well plates and treated with starving medium or medium plus CI, CVI, or FCS. After 6, 12, or 24 h, floating and adherent cells were pooled and lysed with 500 µl of lysis buffer (1% SDS, 10 mM Tris, pH 7.4) for 30 min at 4 °C. Lysed cells were transferred to 1.5-ml Eppendorf tubes and centrifuged at 13,000 rpm for 20 min to separate low molecular weight DNA (oligonucleosome-sized fragments derived from apoptotic cells) from high molecular weight DNA (from viable cells). 400 µl of the supernatant containing oligonucleosomes were carefully transferred to another Eppendorf tube and diluted 1:5 with lysis buffer. A 100-µl aliquot was used for the ELISA, which was performed according to the manufacturer’s instructions (Cell Death Detection ELISA; Roche Molecular Biochemicals). In brief, antibodies against histones were coated onto 96-well, 100-µl lysis samples containing oligonucleotides, and histones were added and incubated for 1 h. After washing, the wells were incubated with anti-double strand DNA antibody coupled to peroxidase for 1 h. After reaction with peroxidase substrate, absorbance was measured at 405 nm using an ELISA reader. Background values (lysis buffer alone) were subtracted, and results are shown as enrichment factor (enrichment factor = OD (optical density) of the sample (apoptotic cells) divided by the OD of the corresponding control (viable cells)).

**FACS Analysis**—1.5 × 10⁶ freshly set cells were treated with medium, CI, CVI, or FCS for 24 or 48 h as described before. After trypsinization, cells were adjusted to the same cell number, washed with 10 ml of PBS, fixed in 70% ethanol for 30 min at −20 °C, and suspended in 500 µl of 100 mM Tris, pH 7.4, containing 10 mM EDTA, 0.5% SDS, 10 mM Tris-HCl, pH 7.4). DNA was sheared with an insulin needle before centrifugation at 13,000 rpm for 20 min, and protein concentration in each sample was determined using the BCA method (Pierce). Equivalent amounts of protein were removed, and volumes were adjusted to 20 µl. After the addition of 2X sample buffer, the aliquots were boiled for 5 min and subjected to 12% SDS-PAGE.

**Western Blot Analysis**—Trypsinized and washed cells were centrifuged at 800 × g and lysed by the addition of 100 µl of lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4). DNA was sheared with an insulin needle before centrifugation at 800 × g. Membrane fragments were removed by centrifugation at 13,000 rpm for 10 min, and protein concentration in each sample was determined using the BCA method (Pierce). Equivalent amounts of protein were removed, and volumes were adjusted to 20 µl. After the addition of 2X sample buffer, the aliquots were boiled for 5 min and subjected to 12% SDS-PAGE.

**DNA Laddering**—After a 24-h treatment with medium, CI, CVI, or 10% FCS, cells were trypsinized and adjusted to equal cell number (1.5 × 10⁶). The Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals) was used according to the manufacturer’s instructions, leading to isolation of oligonucleosomes. The isolated DNA in each group was electrophoresed on a 1.5% agarose gel at 75 V for 1.5 h, stained with ethidium bromide, and visualized via a UV light source (302 nm). As a positive control, apoptotic DNA from HL60 cells (promyelocytic leukemia) was used.

**Cell Death Detection ELISA**—4 × 10⁶ cells were plated in six-well plates and treated with starving medium or medium plus CI, CVI, or FCS. After 6, 12, or 24 h, floating and adherent cells were pooled and lysed with 500 µl of lysis buffer (1% SDS, 10 mM Tris, pH 7.4) for 30 min at 4 °C. Lysed cells were transferred to 1.5-ml Eppendorf tubes and centrifuged at 13,000 rpm for 20 min to separate low molecular weight DNA (oligonucleosome-sized fragments derived from apoptotic cells) from high molecular weight DNA (from viable cells). 400 µl of the supernatant containing oligonucleosomes were carefully transferred to another Eppendorf tube and diluted 1:5 with lysis buffer. A 100-µl aliquot was used for the ELISA, which was performed according to the manufacturer’s instructions (Cell Death Detection ELISA; Roche Molecular Biochemicals). In brief, antibodies against histones were coated onto 96-well, 100-µl lysis samples containing oligonucleotides, and histones were added and incubated for 1 h. After washing, the wells were incubated with anti-double strand DNA antibody coupled to peroxidase for 1 h. After reaction with peroxidase substrate, absorbance was measured at 405 nm using an ELISA reader. Background values (lysis buffer alone) were subtracted, and results are shown as enrichment factor (enrichment factor = OD (optical density) of the sample (apoptotic cells) divided by the OD of the corresponding control (viable cells)).

**FACS Analysis**—1.5 × 10⁶ freshly set cells were treated with medium, CI, CVI, or FCS for 24 or 48 h as described before. After trypsinization, cells were adjusted to the same cell number, washed with 10 ml of PBS, fixed in 70% ethanol for 30 min at −20 °C, and suspended in 500 µl of 100 mM Tris (pH 7.4), containing 10 mM EDTA, 0.5% SDS, 10 mM Tris-HCl, pH 7.4). DNA was sheared with an insulin needle before centrifugation at 800 × g. Membrane fragments were removed by centrifugation at 13,000 rpm for 10 min, and protein concentration in each sample was determined using the BCA method (Pierce). Equivalent amounts of protein were removed, and volumes were adjusted to 20 µl. After the addition of 2X sample buffer, the aliquots were boiled for 5 min and subjected to 12% SDS-PAGE.

**Western Blot Analysis**—Trypsinized and washed cells were centrifuged at 800 × g and lysed by the addition of 100 µl of lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4). DNA was sheared with an insulin needle before centrifugation at 800 × g. Membrane fragments were removed by centrifugation at 13,000 rpm for 10 min, and protein concentration in each sample was determined using the BCA method (Pierce). Equivalent amounts of protein were removed, and volumes were adjusted to 20 µl. After the addition of 2X sample buffer, the aliquots were boiled for 5 min and subjected to 12% SDS-PAGE.
temperature. Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia Biotech). After stripping in 0.1 M glycine-HCl, pH 2, for 20 min at room temperature, membranes were reprobed with appropriate primary and secondary antibodies. Band intensities were analyzed by densitometry. Results are shown as percentage of the control (10% FCS) after adjustment of band intensity to that of β-actin, which served as internal control.

RESULTS

Soluble Type VI Collagen Inhibits Apoptosis in Serum-starved Fibroblasts—Previous studies have shown that CVI strongly induces cellular attachment and spreading (24, 29, 32). To test the hypothesis that soluble CVI inhibits programmed cell death, we induced apoptosis in transformed and nontransformed fibroblasts by serum withdrawal (33). HT1080 and Balb 3T3 cells were synchronized over 24–48 h by culturing in Dulbecco’s modified Eagle’s medium containing no FCS, trypsinized thereafter, and seeded for the different assays as described under “Experimental Procedures.” After plating, cells were treated with plain medium or 20 mg/liter pepsin-solubilized CI as negative controls, with 10% FCS as positive control or with 20 mg/liter CVI. Initial experiments included exposure of cells also to soluble collagens III, IV, V, or XIV; to fibronectin; or to laminin, which were ineffective in inhibiting apoptosis and of which CI was chosen as negative control for all subsequent experiments. After an additional 24–48 h, cells were harvested and subjected to several complementary assays to assess apoptosis.

The morphological features of apoptosis (i.e., cell shrinkage and nuclear condensation) were evident with medium alone or CI, whereas cells exposed to CVI or FCS remained well spread and viable after 24 h of treatment (Fig. 1). Initially, cells in each group adhered and spread to an equal extent, but, whereas cells exposed to medium or CI detached after 24 h, cells treated with 10% FCS or CVI remained well spread for up to 96 h (data not shown).

After 24 h the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay that highlights apoptotic cells was clearly positive in 15–20% of cells treated with medium alone or CI, contrasting with less than 2% positive cells in the cultures treated with CVI or 10% FCS (Fig. 2).

DNA laddering (degradation into multiples of 180-base pair-long fragments) has been considered as one of the most characteristic signs of apoptotic cell death (1). Serum-starved cells were plated and treated as described before. When the isolated DNA was loaded onto a 2% agarose gel, the cells exposed to medium and CI exhibited typical laddering, whereas no DNA fragmentation was visible in the presence of CVI or 10% FCS (Fig. 3).

To quantify apoptotic cell death and to better delineate its time course, we used the cell death detection ELISA (Fig. 4) and FACS analysis (Fig. 5). Both methods confirmed the antiapoptotic effect of soluble CVI at 20 mg/liter (60 nmol/liter), which was equipotent to 10% FCS. FACS analysis showed that after 24 h of treatment with CVI or 10% FCS, less than 1% of the cells could be identified as apoptotic compared with 21 and 15% in the medium and CI groups, respectively. After 48 h, the percentage of apoptotic cells rose to 47% in the medium and CI group and increased moderately in the CVI (12.5%) and 10% FCS (5.5%) groups, respectively. Additionally, compared with plain medium or CI, a higher number of cells in the CVI and 10% FCS groups were driven into the G2 and S phase of the cell cycle, as evidenced by doubled or increased DNA content (Fig. 5). After 48 h of treatment, 18 and 19%, respectively, of the cells treated with CVI or 10% FCS could be detected in G2 compared with 3.8 and 5% in the medium and CI groups.

Soluble CVI Is More Effective than Immobilized CVI in Preventing Apoptosis—In order to compare the antiapoptotic effect of added soluble CVI with that of immobilized CVI, we seeded HT1080 cells on the nonadhesive substrates poly-HEMA (Fig. 6B) or BSA (Fig. 6C) or on plastic, CI, or CVI, followed by the addition of soluble CI, CVI, plain medium, or FCS (Fig. 6A).
Collagen Type VI Inhibits Fibroblast Apoptosis

After 24 h, propidium iodide-stained cells were subjected to FACS analysis. Here, soluble CVI was twice as efficient in preventing apoptosis as coated CVI and 4 times more efficient than coated or soluble CI (Fig. 6A). These experiments again showed that soluble CVI is almost as potent as 10% FCS in preventing apoptosis with 8 and 6% apoptotic cells, respectively.

CVI Prevents Apoptosis via a Partially Spreading and β3-Integrin-independent Mechanism—Attachment and spreading were decreased by coating the wells with the nonadhesive substrata poly-HEMA (Fig. 6B) or BSA (Fig. 6C). Since anchorage-dependent cells undergo apoptosis under conditions that prevent attachment and spreading (14), we next investigated whether the anti-apoptotic effect of type VI collagen might be due to promoting attachment and spreading. Cells plated on poly-HEMA showed no spreading in any treatment group, and 40% of cells were apoptotic when treated with plain medium or CI after 24 h compared with 20 and 10% in the CVI-treated and 10% FCS groups, respectively. The survival-promoting effect of soluble CVI on cells plated onto BSA was even more pronounced: less than 1% of the cells died compared with 20–25% apoptotic cells treated with CI or plain medium. These results suggest that spreading-independent mechanisms contribute to the anti-apoptotic effect of soluble CVI.

When β3-integrin function was blocked with monoclonal antibody P4C10 shortly before the addition of CI or CVI, cell spreading was inhibited. Under these conditions, the ratios of apoptotic cells versus cells in G1 were 3.0, 1.2, and 0.7 for plain medium, collagen I, and collagen VI, respectively, compared with complete suppression of apoptosis by FCS (Fig. 6D). This indicates an important but not exclusive role of β3-integrins in antiapoptotic signals transduced by CVI.

Proapoptotic Bax Is Down-regulated by Soluble CVI—to study whether soluble CVI may be regulating key proapoptotic or antiapoptotic proteins, such as Bax and Bcl-2 (4), we determined the levels of these two proteins by quantitative Western blot analysis. The ratio of Bax/Bcl-2 is thought to be crucial for cell survival. Fig. 7 illustrates that in HT1080 cells, relative to the internal standard β-actin, Bcl-2 levels remained unaltered upon treatment with CVI or 10% FCS compared with medium or CI, whereas in Balb 3T3 cells, 10% FCS but not CVI caused a 3-fold up-regulation of Bcl-2 (data not shown). In contrast, CVI clearly down-regulated Bax 2–3-fold in both cell lines. This effect was comparable with the 3-fold down-regulation of Bax observed with 10% FCS compared with medium alone or CI. Thus, in HT1080 cells, the Bax/Bcl-2 ratio was decreased 3-fold upon CVI treatment, thus favoring cell survival.

Soluble CVI Induces Cell Cycle Progression through Up-regulation of Cyclins A, B, and D1—Cyclins A, D, and E have been reported to be regulated by a coordinated stimulation of growth factors and ECM molecules in adhesion-dependent cells (34). Induction of cell cycle progression through up-regulation of specific cyclins by ECM molecules alone has not been reported. Therefore, we analyzed the expression of cyclins A, B, and D1 after the addition of various stimuli including CVI. Expression of the anchorage-dependent cyclin A is up-regulated during S phase, and cyclin B controls cell cycle progression into G2 (35). Similarly, cyclin D1 controls entry into the G1-S phase in many cell lines, including fibroblasts, but fails to do so in the absence of ECM signaling (36). Fig. 8 shows that CVI alone up-regulated cyclins A, B, and D1 2–3-fold compared with plain medium or CI, comparable with cells treated with 10% FCS. These results are in full agreement with our cell cycle analysis by FACS scan, which showed that only in the presence of CVI or FCS had a significant percentage of cells progressed to G2 (Fig. 5).

Discussion

Adhesion of cells to several ECM proteins can prevent apoptosis under conditions of nutrient and growth factor deficiency. A recent report showed that the soluble, ECM-derived β3-integrin ligand collagen I can rescue detached and serum-starved neuronal cells from apoptosis (37). Here, by using complementary methods, we could demonstrate that soluble, contrary to immobilized, CVI prevents apoptosis in serum-starved HT1080 and Balb 3T3 cells. The antiapoptotic effect of CVI is comparable with that provided by 10% fetal calf serum and far more potent than that contributed by single, classical growth factors for mesenchymal cells. Furthermore, soluble CVI induced a 2–3-fold down-regulation of the proapoptotic Bax, again comparable with 10% FCS, whereas expression of Bcl-2 was unaffected. In line with our previous observations of the strong activation of DNA-synthesis by soluble CVI (30), its antiapoptotic effect was accompanied by a 2–3-fold up-regulation, compared with medium control, of cyclins A, B, and D1, which are required for progression of the cell cycle from G1 to G2. This up-regulation reached 70–80% of cells cultured in 10% FCS.

When offered CVI as adhesive substrate, the viability of corneal cells is enhanced 2-fold compared with a matrix of CI (26). While our data support these findings for immobilized CVI, the soluble form of CVI, compared with soluble collagen I, is even more potent in preventing apoptosis in HT1080 and 3T3 cells.

One explanation for the more pronounced antiapoptotic effect of soluble CVI may be the creation of a pericellular microfilamentous CVI network due to in vitro polymerization (38) that resembles the pericellular CVI matrix in vivo and in—
Increases interactions with the cellular CVI receptors. Such interactions can subject cells to increased tension and are accompanied by enhanced spreading (39). The degree of spreading has been shown to correlate with cell survival and proliferation (40). Besides determining proliferation and apoptosis via cell shape alterations, generation of tensile stress may alter responsiveness to growth factors (41). Thus, fibroblasts that are cultured in collagen matrices respond to PDGF signaling with DNA synthesis, while the same cells fail to respond to the growth factor when stress is relaxed in a collagen gel (41). However, when cells were plated on nonadhesive surfaces, we observed no spreading after the addition of soluble CVI, despite a potent antiapoptotic effect, suggesting the existence of spreading-independent pathways of CVI-induced signal.

![Graph showing time course of apoptotic cell death in serum-starved HT1080 cells (cell death detection ELISA).](image1)

**FIG. 4.** Time course of apoptotic cell death in serum-starved HT1080 cells (cell death detection ELISA). $4 \times 10^5$ cells were cultured in six-well plates and treated with plain medium, CI or CVI (final concentration 20 mg/liter), or 10% FCS. After 6, 12, or 24 h, cells were lysed, and low molecular weight oligonucleosomal DNA (from viable cells) was detected by a sandwich ELISA with antibodies against histones and DNA. Background OD values (lysis buffer alone) were subtracted, and results are shown as enrichment factor as proposed by the manufacturer (enrichment factor = OD (optical density) of the sample (apoptotic cells) divided by the OD of the corresponding control (viable cells, FCS group)). Cells were exposed to the following: M, starving medium; CI, collagen I (20 mg/liter); CVI, collagen VI (20 mg/liter); FCS, 10% FCS. Apoptosis started between 6 and 12 h after serum starvation, reaching an enrichment factor of 9 and 5 after 24 h in the medium alone and CI-treated group, respectively. Treatment with CVI as well as treatment with 10% FCS resulted in strong inhibition of apoptosis during all treatment periods (enrichment factor 1–2). Shown are means ± S.E. of three experiments.

![Graph showing cell cycle analysis of HT1080 cells exposed to starving medium, collagens, or FCS.](image2)

**FIG. 5.** Cell cycle analysis of HT1080 cells exposed to starving medium, collagens, or FCS. Serum-starved HT1080 cells were treated with medium alone, CI, CVI (both at 20 mg/l), or 10% FCS for 24 or 48 h. After fixation in 70% ethanol and RNase digestion, cellular DNA was stained with propidium iodide and subjected to FACS analysis. The integrated area of cellular DNA content was calculated as percentage of cells in G0-G1, G2-M, S phase transition, or apoptosis (hp, hypoploid cells). Shown is a representative of three independent experiments.
transduction. Whereas the integrins α2β1 and α1β1 are receptors for native, triple helical CVI, and αvβ3, αvβ5, and αIIbβ3 recognize denatured CVI, they all promote cell migration and spreading after ligation of CVI (29). A nonintegrin CVI receptor, the chondroitin sulfate proteoglycan NG2 (42–45), has been shown to colocalize with the PDGF α receptor. Interestingly, PDGF α receptor signaling is disrupted by NG2 down-regulation, suggesting a cross-talk between CVI and PDGF receptors (46, 47).

In accordance with these findings and previous reports (26, 30), the effects of CVI on proliferation and apoptosis were not solely dependent on β1-integrins, since integrin-blocking antibodies only partially influenced CVI-induced proliferation or apoptosis as shown in Fig. 6D, suggesting that NG2 could be an important proliferation-inducing and antiapoptotic CVI receptor (42–45).

Searching for proteins that are involved in the CVI-mediated prevention of apoptosis, we studied the expression of antiapoptotic Bcl-2 and proapoptotic Bax, the ratio of which determines the susceptibility to programmed cell death in various cell types (48, 49). CVI suppressed Bax up-regulation upon serum withdrawal to a similar degree as treatment with 10% FCS. In addition to growth factors, ECM-receptor interactions

**FIG. 6.** Cell survival on antiadhesive or adhesive surfaces. 10⁶ serum-starved HT1080 cells were seeded into uncoated (–) six-well plates or plates coated with collagens (CI and CVI) (A), poly-HEMA (pHema) (B), and 1% BSA in PBS (BSA) (C). D, cells were added to uncoated wells 2 h after the addition of β1-integrin receptor blocking antibody P4C10. Wells were then immediately treated with medium alone (–), soluble CI (sCI) or CVI (sCVI), or 10% FCS (FCS). After 24 h, cells were subjected to FACS analysis as shown in Fig. 5. The area under the shoulder left of the peak that represents the G₀-G₁ fraction of the FACS histogram in D (hypopl, hypoploid cells) was taken as the percentage of apoptotic cells. The results shown are means ± S.E. or means of three or four independent experiments.

**FIG. 7.** Expression of Bax and Bcl-2 protein in collagen VI-treated HT1080 cells. HT1080 cells were treated with medium alone, CI, CVI, or 10% FCS for 24 h. Cell lysates were adjusted to equal protein contents, run on a 12% SDS-PAGE, and blotted onto nitrocellulose, followed by sequential incubation with polyclonal rabbit anti-human Bax, monoclonal mouse anti-human Bcl-2, or polyclonal rabbit anti-human β-actin. Membranes were developed with anti-mouse-IgG coupled to peroxidase and detected with the ECL chemiluminescence reagent. After stripping in acidic glycine buffer, membranes were reprobed with appropriate primary and secondary antibodies. Bands were analyzed by densitometry, and results are shown as percentage of the 10% FCS-treated control, normalized to cellular β-actin content. M, starving medium; CI, collagen I (20 mg/liter); CVI, collagen VI (20 mg/liter); FCS, 10% FCS. B, ratio of Bax/Bcl-2 for HT1080 cells. Shown is a representative example of four independent experiments.
Collagen Type VI Inhibits Fibroblast Apoptosis

activating protein kinase, extracellular signal-regulated kinase-2, a major second messenger in mitogenesis, is activated 20-fold after exposure of HT1080 cells to CVI (51), the observed up-regulation of cyclin D1 might be linked to mitogen-activated protein kinase activation (53). We therefore hypothesize that CVI has intrinsic growth factor-like properties.

A prominent matrix of CVI is found around fibroblasts (16), neural crest (32), and hematopoietic stem cells (20), suggesting a role of this collagen for mesenchymal cell differentiation during development. Increased expression of CVI is found in the ECM of melanomas or gliomas (27, 28) or in chronic fibrotic conditions (54, 55). Thus, CVI-content is increased 10-fold in fibrotic livers, and CVI serum levels are highly elevated in adults and children with advanced renal and hepatic fibrosis (55–57). Therefore, enhanced proteolysis and release of CVI during continuous inflammation and matrix turnover could trigger mesenchymal cell proliferation in an autocrine and paracrine manner.

Considering the potential of CVI to serve as a survival factor for fibrogenic cells, novel strategies for antifibrotic treatment can be envisaged. Thus, CVI-receptor-recognizing peptides may specifically target and antagonize activated myofibroblasts, which are the main producers of excess ECM in fibrosis of the liver, kidneys, lungs, and arteries. Recent in vivo experiments show that after intravenous injection more than 50% of a cyclic peptide with specificity for a CVI receptor (58) reaches activated hepatic stellate cells, the major fibrogenic cells in liver (59).

Acknowledgments—We thank Renate Ackermann and Monika Schmid for expert technical assistance and Dr. S. Rosewicz, K. Detjens, and M. Welser for support in FACS analysis.

REFERENCES

1. Wyllie, A. H. (1993) Br. J. Cancer 67, 205–208
2. Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Waking, A. E., Walker, P. R., and Sikorska, M. (1993) EMBO J. 12, 3679–3684
3. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
4. Green, D. (1998) Cell 94, 695–690
5. Kroemer, G. (1997) Nat. Med. 3, 614–620
6. Marzo, I., Brenner, C., Zammarsi, N., Jurgenesmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuzawa, S., Reed, J. C., and Kroemer, O. (1998) Science 281, 1297–1301
7. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
8. Gajewski, T., and Thompson, C. B. (1996) Cell 87, 589–592
9. Boudreau, N., Werb, Z., and Bissell, M. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3509–3513
10. Meredith, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8856–8860
11. Roskelley, C. D., Srebrow, A., and Bissell, M. J. (1995) Curr. Opin. Cell Biol. 7, 736–747
12. Frisch, S. M., and Francis, H. (1994) J. Cell Biol. 124, 619–626
13. Bussolati, B., Reed, J. C. (1994) Cell 77, 471–478
14. Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. (1993) Mol. Biol. Cell 4, 953–961
15. Aplin, A. E., Howe, A., Alahari, S. K., and Juliano, R. L. (1998) Pharmacol. Rev. 50, 197–203
16. Bruns, R. R., Press, W., Engelw, E., Timpl, R., and Gross, J. (1986) J. Cell Biol. 103, 393–404
17. Lorial, O., Clement, B., Schuppan, D., Rescan, P. Y., Rissel, M., and Guillozoa, A. (1992) Gastroenterology 102, 880–887
18. Hatamochi, A., Aumailey, M., Mauch, C., Chu, M. L., Timpl, R., and Krieg, T. (1989) J. Biol. Chem. 264, 3494–3499
19. Kuo, H. J., Maisen, C. L., Keene, D. R., and Glanville, R. W. (1997) J. Biol. Chem. 272, 26522–26529
20. Kiely, C. M., Whitaker, S. P., Grant, M. E., and Shuttlesworth, C. A. (1992) J. Biol. Chem. 267, 1740–1748
21. Klein, G., Muller, C. A., Tillet, E., Chu, M. L., and Timpl, R. (1995) Blood 86, 1740–1748
22. Bidanset, D. J., Guidry, C., Rosenberg, L. C., Choi, H. U., Timpl, R., and Hook, J. (1992) J. Biol. Chem. 267, 5250–5256
23. Holm, N., Yamasato, K., Nuteis, K., Vrey, I., Dernm, H., and Vermynen, J. (1997) Biochem. J. 324, 185–191
24. Brown, J. C., Mann, K., Wiedemann, H., and Timpl, R. (1993) J. Cell Biol. 120, 557–561
25. Doane, K. J., Yang, G., and Birk, D. E. (1992) Exp. Cell Res. 200, 490–499
26. Howell, S. J., and Doane, K. J. (1998) Exp. Cell Res. 241, 230–241
27. Han, J., Daniel, J. C., and Pappas, G. D. (1995) Cancer Lett. 86, 127–132
28. Daniels, K. J., Boldt, H. C., Martin, J. A., Gardner, L. M., Meyer, M., and Folfberg, R. (1996) Lab. Invest. 75, 55–66
29. Pfaff, M., Aumailey, M., Specks, U., Knolle, J., Zerwes, H. G., and Timpl, R.
Collagen Type VI Inhibits Fibroblast Apoptosis