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The terminal complement complex inhibits apoptosis in vascular smooth muscle cells by activating an autocrine IGF-1 loop

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ABSTRACT

Two counteracting processes determine accumulation of human vascular smooth muscle cells (SMCs) in atherosclerotic lesions: cell proliferation and apoptosis. SMCs synthesize insulin-like growth factor-1 (IGF-1), which potently inhibits apoptosis. The terminal complement complex C5b-9 interacts with SMCs in early human atherogenesis. In this study, we investigated whether C5b-9 may activate the IGF-1 system in SMCs, resulting in the inhibition of SMC apoptosis. C5b-9 generation on SMCs *in vitro* markedly reduced CD95-mediated apoptosis as assessed by flowcytometric analysis of annexin V binding and in caspase 3 assays. C5b-9 induced both significant IGF-1 release and up-regulation of IGF-1 binding sites in SMCs. Immunoneutralization of IGF-1 with a monoclonal IGF-1 antibody abolished the antiapoptotic effects of C5b-9. We conclude that C5b-9 inhibits apoptosis in SMCs by inducing an autocrine IGF-1 loop. This mechanism may contribute to the accumulation of SMCs in early human atherosclerotic lesions.

Key words: arteriosclerosis • cell death • inflammation • C5b-9 • growth factors

Atherosclerotic lesion formation results, at least in part, from excessive growth of smooth muscle cells (SMCs) (1–3). The accumulation of SMCs in the arterial wall depends on the balance between cell death and proliferation. Thus, apoptosis is considered to be a major regulative mechanism determining SMC accumulation during atherogenesis (4–7). Interestingly, early lesions show little apoptosis. However, the level of apoptosis increases with plaque formation (4).

One known factor influencing apoptosis of SMCs is insulin-like growth factor (IGF-1). Apoptosis has been shown to be inhibited by IGF-1 in SMCs through IGF-1 receptors modulated

by IGF binding proteins (IGFBPs). Several studies suggest that the IGF system is intricately involved in the pathogenesis of atherosclerosis (2).

In this context, the terminal complement complex C5b-9 might be of interest for several reasons: First, C5b-9 extensively colocalizes with SMCs during early atherogenesis (8–9), whereas in advanced lesions, C5b-9 is mainly confined to macrophages; second, sublytic C5b-9 is antiapoptotic for oligodendrocytes and Schwann cells (10–12); and third, sublytic C5b-9 induces release of cytokines/chemokines (13) and growth factors (14) from nucleated cells.

In this study, we examined whether generation of sublytic C5b-9 on SMCs *in vitro* influences apoptosis via interaction with the IGF system.

MATERIALS AND METHODS

Cell culture

SMCs were isolated from tissue explants from surplus segments of human mammary arteries obtained during coronary artery bypass surgery using the explant method (13, 15). Cells were identified as SMCs by anti-α-actin stain. The secondary cultures of human SMCs were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 20% fetal calf serum (FCS) (PAA, Linz, Austria), fed once every 3 days, and split at a ratio at 1:2 upon reaching confluence. Subcultures 5 through 8 were used and cultured in DMEM containing 20% FCS and antibiotics. Control experiments were performed using commercially available human coronary artery smooth muscle cells (BioWhittaker, Walkersville, MD) (passage 3–5).

C5b-9 formation on SMCs

C5b-9 formation on SMCs was performed by a "reactive lysis" mechanism (16, 17). In brief, C5b6 was isolated from yeast-activated acute-phase serum by anion exchange and gel filtration. The dose of C7 (Sigma, St. Louis, MO) equivalent to the dose of C5b6 was determined by titration with guinea pig erythrocytes. Sublytic C5b-9 complexes were generated on SMCs by adding C5b6 to SMCs in monolayer culture. After 15 min, equivalent doses of C7 were added. A surplus of C8 and C9 (Sigma) was added 15 min later. One minimum hemolytic dose (MHD) was defined as the amount of C5b-9 needed to produce lysis of 50 μl 1% erythrocytes. C7 activity ranging from 6.9 to 7.8 × 10^6/U/mg per cm^2 cell lane with equivalent C5b6 and C8/C9 was found to be sublytic for SMCs. This dosage corresponded to 100 MHD C5b-9. Lysis was assessed by cell morphology and Trypan blue uptake.

SMC apoptosis

Apoptosis assays for SMCs were performed as described previously (18, 19). To sensitize SMCs for apoptotic stimuli, we treated cells that were 75% confluent with IFN-γ (500 U/ml, R&D Systems, Minneapolis, MN), IL-1β (100 U/ml, R&D Systems), and tumor necrosis factor (TNF)-α (Sigma) for 24 h following established protocols (17, 18). Cells were washed three times with phosphate-buffered saline (PBS) and then stimulated with mouse anti-human CD95 monoclonal antibody CH11 (Immunotech, Marseille, France) at a concentration of 500 ng/ml in

DMEM/0.2% FCS. Alternatively, apoptosis in SMCs was induced by 15 min UV light treatment (280–320 nm). Control experiments included C5b-9 substitution by human recombinant IGF-1 (R&D Systems) at a concentration of 10^{-10} M. Exposure of phosphatidylserine on cell surfaces was assessed with the ApoAlert Annexin V Kit (Clontech, Palo Alto, CA) by flow cytometry analysis in a fluorescence activated cell sorter (FACS) (Becton Dickinson, Franklin Lakes, NJ), using the CellQuest software system (20). A monoclonal IGF-1 antibody (Upstate Biotechnology, Lake Placid, NY) was used for immunoneutralization of IGF-1 as described previously (21). The inhibitory effect of antibody treatment was time- and concentration-dependent. A maximum effect was achieved at an antibody concentration of 25 μg/ml with a 24-h preincubation period.

Caspase 3 activity assays were performed in a fluorometric immunsorbend enzyme assay. For this assay, cellular lysates were captured in microtiter plates by a monoclonal antibody against caspase 3 (Roche, Indianapolis, IN). Following the washing steps, caspase 3 substrate Ac-DEVD-AFC (Roche) was added. Due to proteolytical cleavage of the substrate, free fluorescent AFC was generated proportional to the amount of activated caspase 3. Free AFC was then determined fluorometrically at λ_{max}=505 nm.

Analysis of cell supernatants

The amount of secreted IGF-1 and IGF-2 was determined by specific radioimmunoassays (DSL) (22). IGF binding protein (IGFBP) release was assessed by Western ligand blot analysis. To further characterize IGFBP release in SMCs, Western ligand blot analysis of conditioned media was also performed (22). In brief, 500 μl of conditioned media were lyophilized, dissolved in 50 μl of sample buffer [50 mM Na_2HPO_4, pH 7.0; 1% (w/v) sodium dodecyl sulfate (SDS); 50% (w/v) glycerin], and boiled (5 min), and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), using the Mini Protean II™ system (Bio-Rad, Hercules, CA). Separated proteins were transferred to a nitrocellulose membrane (Millipore, Bedford, MA). The blots were blocked with 1% fish gelatin and incubated with [^{125}I]IGF-II (10^6 cpm per blot). IGFBPs were visualized on Phosphor-Imager Storm (Molecular Dynamics, Sunnyvale, CA). All hybridization and washing steps were performed at 4°C.

Receptor binding studies

SMCs were treated with either sublytic C5b-9 or buffer (control) for 18 h, and competition-inhibition binding assays were performed with ^{125}I-IGF-1 (10 pM) and increasing concentrations of unlabeled IGF-1 (0.01–100 nM) (20–22). The number of binding sites per cell and binding affinity (K_d values) were estimated by computer-assisted Scatchard analysis (23).

Isolation of total RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) for IGF-1

Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany). Amplification of IGF-1 and GAPDH was performed using the Omniscript RT kit (Qiagen). Reverse transcription was performed for 60 min at 37°C. Taq PCR Master Mix kit (Qiagen) was used for amplification of PCR products. To quantitatively assess GAPDH, we calibrated PCR by amplification of defined amounts of template GAPDH cDNA cloned into pGEM-T vector (Promega, Madison,

WI). The proportion of synthesized RT-PCR products remained constant with 25, 30, and 35 cycles of PCR. Therefore, 30 cycles were chosen for further analysis. PCR was performed for 30 cycles each at 94°C (3 min), 56°C (40 s), and 72°C (1 min). The forward primer for IGF-1 was 5'-CGT GGA TGA GTA CTA CTT CC-3' and the reverse primer was 5'-AAG AGG TAA CTC GTG CAG AG-3'. The forward primer for GAPDH was 5'- ACG GAT TTG GTC GTA TTG GGC -3' and the reverse primer was 5'-CTC CTG GAA GAT GGT GAT-3'.

Flow cytometry of IGF-1 receptors

Cells were stained for IGF-1 receptors, using monoclonal R-PE-conjugated anti-IGF-1 receptor antibody (20 μl/10^6 cells; PharMingen, Palo Alto, CA). Cells were analyzed by flow cytometry, using a fluorescence-activated cell sorter (20). The background gate was set up using unstimulated SMCs (5% positive cells). Anti-mouse isotype-matched antibodies were used as controls.

RESULTS

C5b-9 inhibits CD95-mediated induction of apoptosis

To determine differences in the amount of apoptosis, we performed annexin V binding experiments. Stimulation of SMCs was accomplished by preincubation with sublytic C5b-9 for 4 h followed by incubation with CH11 for 0, 1, 3, 6, 12, and 24 h. Flow cytometric analysis revealed a time-dependent enhancement of annexin V binding (Fig. 1). Preincubation of SMCs with sublytic C5b-9 inhibited this shift in annexin V binding. Control cells pretreated with C5b6, C8, and C9 lacking C7 underwent apoptosis in a similar rate as SMCs without complement, thus indicating that the antiapoptotic effects of C5b-9 require complete complex formation. Analogous antiapoptotic effects of sublytic C5b-9 were observed in annexin V binding experiments, using commercially available SMCs (data not shown).

Kinetic studies of caspase 3 activity after C5b-9 and CH11 stimulation at 0, 24, 48, and 72 h (Fig. 2A) confirmed the antiapoptotic effect of C5b-9 on CD95-mediated programmed cell death. Stimulation of SMCs with CH11 alone led to a significant increase in caspase 3 activity, whereas the coincubation with CH11 and C5b-9 significantly ($P<0.05$) decreased caspase 3 activity even below the activity level of unstimulated cells (Fig. 2A). In addition, the number of C5b-9 complexes formed on the membrane of SMCs had an effect on the antiapoptotic properties of C5b-9. Ten MHD of C5b-9 had no antiapoptotic effect, whereas 500 MHD C5b-9 inhibited apoptosis at the same rate as 100 MHD C5b-9 ($P<0.05$), (Fig. 2A).

C5b-9 inhibits UV-mediated induction of apoptosis

To test whether the observed antiapoptotic effect was specific to CD95-mediated apoptosis, we induced apoptosis in SMCs by an alternative method using UV light (Fig. 2B). Pretreatment of the SMCs with sublytic C5b-9 also significantly reduced caspase 3 activity after UV light treatment ($P<0.05$).

C5b-9 increases IGF-1 secretion

To measure the direct effect of C5b-9 on IGF-1 secretion, we stimulated SMCs with sublytic C5b-9 and measured the IGF-1 accumulation in the supernatant. The supernatant was analyzed 0, 6, 12, 24, and 48 h after stimulation with C5b-9. Preincubation with C5b-9 significantly increased IGF-1 secretion from SMCs in a time-dependent manner, with maximum effect achieved at 24 h (Fig. 3A). The release of IGF-2 was markedly higher than that of IGF-1 but was not significantly influenced by C5b-9 (Fig. 3B). Western ligand blot analysis of conditioned media revealed the presence of different IGFBPs (Fig. 3C). IGF binding activities were found as a band doublet between 36 and 45 kDa, and weak singular bands at 32 and 24 kDa. However, no regulative function of C5b-9 could be attributed to any of these binding activities. To investigate whether IGF-1 secretion is due to an increase in RNA, we performed semiquantitative RT-PCR of IGF-1 (Fig. 3D). Comparison of the IGF-1 mRNA amount in cells preincubated with sublytic C5b-9 and control cells revealed no difference in mRNA amount, indicating that no *de novo* synthesis of IGF-1 mRNA is induced by C5b-9 stimulation.

C5b-9 increases the number of IGF-1 receptors and IGF-1 binding sites on SMCs

We used both flow cytometry and IGF-1 binding assays to address whether C5b-9 affects the number of IGF-1 receptors and binding sites on SMCs. Flow cytometric analysis of IGF-1 receptors revealed a time-dependent shift of gated cells from 5.1% (0 h) to 20.9% (24 h) (Fig. 4A). Analysis of control cells (24 h) without C5b-9 stimulation revealed no significant up-regulation of IGF-1 receptors (data not shown). Incubation with sublytic C5b-9 for 18 h induces a significant increase in the amount of specifically bound IGF-1 ($P<0.01$ vs. control) as shown by ^{125}I-IGF-1 binding assays ($n=4$; Fig. 4B). Linear Scatchard plots (Fig. 4C) indicate that this was due to an increase in IGF-1 receptor number (101,300 ± 18,300, control vs. 203,000 ± 46,500, C5b-9; $P<0.02$) without changes in binding affinity (K_d values: 0.63 ± 0.33 nM, control vs. 0.85 ± 0.4 nM, C5b-9).

IGF-1 is involved in antiapoptotic properties of C5b-9

We further tested the role of IGF-1 in C5b-9 mediated inhibition of apoptosis. SMCs were stimulated with sublytic C5b-9 and incubated with CH11 for 0, 6, 12, and 24 h. To assess whether IGF-1 may be involved in C5b-9-mediated inhibition of apoptosis, we preincubated SMCs with a monoclonal IGF-1 antibody to immunoneutralize autocrine-released IGF-1 (21). The IGF-1 antibody abolished the antiapoptotic effects of C5b-9 as assayed by annexin V binding (Fig. 5). In control experiments, no apoptotic effect of the IGF-1 antibody was seen when SMCs were incubated with the antibody alone. Furthermore, no additional apoptotic effect was observed in combination with CH11 (data not shown).

IGF-1 is antiapoptotic for SMCs

To evaluate the extent of the antiapoptotic effect of C5b-9 in comparison to IFG-1, we performed further annexin V assays. Substitution of C5b-9 with recombinant IGF-1 inhibited enhancement of annexin V binding following CH11 stimulation of SMCs in a similar manner as C5b-9 preincubation (Fig. 6).

DISCUSSION

In this paper, we demonstrate by annexin V labeling and caspase 3 activity analysis of apoptotic cells that generation of sublytic C5b-9 on SMCs *in vitro* inhibits CD95-mediated apoptosis in these cells. In addition, we demonstrate that the observed antiapoptotic effect is not only limited to CD95-mediated apoptosis; it is dependent on formation of the complete transmembrane pore, as omission of C7 abolishes the regulative function of C5b-9 for SMC apoptosis. Furthermore, we demonstrate that C5b-9 induces both IGF-1 release and up-regulation of IGF-1 binding sites in SMCs, whereas secretion of IGF-2 and IGFBPs was not influenced. Induction of IGF-1 release did not involve IGF-1 *de novo* synthesis as shown by RT-PCR.

Immunoneutralization of endogenously released IGF-1 by a blocking antibody abolished the inhibitory effects of C5b-9 on SMC apoptosis. Furthermore, substitution of C5b-9 with recombinant IGF-1 completely inhibited apoptosis in our cell system. Thus, the endogenously released IGF-1 is a major mediator of the antiapoptotic action of C5b-9. Our findings are in line with former reports on C5b-9- mediated growth factor and cytokine release (14, 24) and inhibition of apoptosis (10–12) in other nucleated cells. However, this study describes for the first time that an autocrine IGF-1 loop causes the antiapoptotic effect of C5b-9 on SMCs. The IGF system is essential in the pathogenesis of atherosclerosis (2). The fact that IGF-1 is involved in C5b-9-mediated inhibition of apoptosis links inflammation to growth factor activation in atherogenesis.

Complement activation is a pathogenic feature both in human and in experimental atherogenesis (25–29) and in other cardiovascular diseases (30). C-reactive protein (CRP), an important cardiovascular risk factor (31), has been identified as a major complement activating molecule in the arterial wall (9, 32–34). The interaction of complement activation products with vascular cells may be of importance in the understanding of inflammatory mechanisms underlying atherosclerosis. As C5b-9 colocalizes with SMCs during early atherogenesis (8, 9), the investigation of direct effects of C5b-9 on SMC proliferation and apoptosis might contribute to the understanding of SMC accumulation in the arterial wall. Interestingly, sublytic C5b-9 has been shown to stimulate SMC proliferation (35). As it is demonstrated in this paper that C5b-9 also inhibits SMC apoptosis, complement activation may be significantly involved in the imbalance between cell death and proliferation (36) observed during atherogenesis. Apoptosis in atherosclerotic lesions is related to plaque stage with early lesions showing little apoptosis and advanced lesions showing apoptotic levels of 2% (4). Furthermore, C5b-9 in advanced lesions is mainly confined to macrophages. Therefore, SMCs in early but not in advanced plaques may be protected against ongoing cell death by the antiapoptotic effects of C5b-9 (4, 8).

Apoptosis in atherosclerotic lesions can also be induced by other factors intimately involved in atherogenesis, such as oxidized LDL (37). Future research may investigate whether C5b-9 also inhibits apoptosis mediated by these molecules.

Inhibition of the complement system may be a potential new therapeutic option for the treatment of atherosclerosis. Additional studies have to be undertaken to investigate the mechanisms by which C5b-9 mediates IGF-1 release and up-regulation of IGF-1 binding sites in SMCs.

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Fig. 1

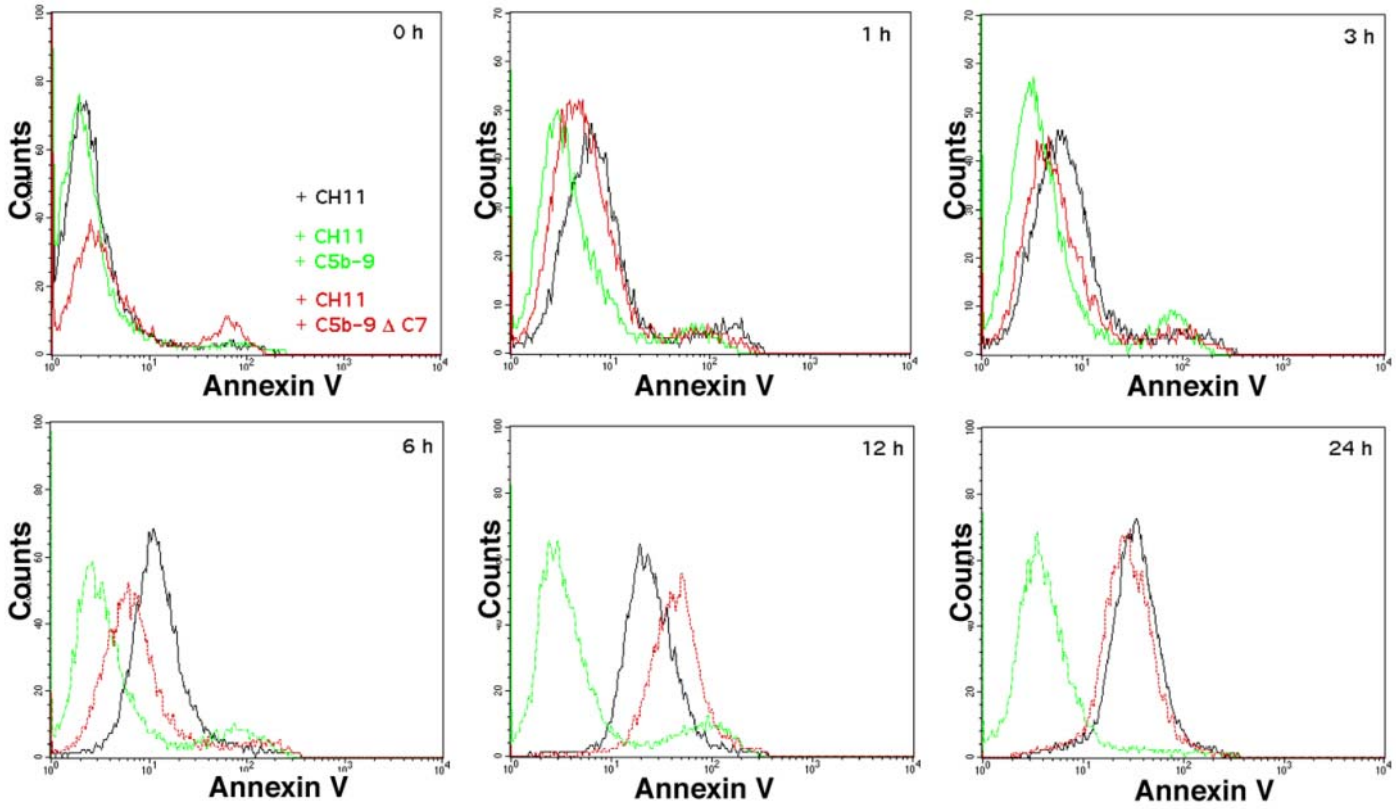

Figure 1. SMC apoptosis. Time-dependent enhancement of annexin V binding following CH11 stimulation of SMCs. Preincubation of SMCs with sublytic C5b-9 (green) inhibits shift in annexin V-binding. SMCs pretreated with C5b6, C8, and C9 lacking C7 (red) underwent apoptosis at a similar rate as SMCs without complement (black).

Fig. 2

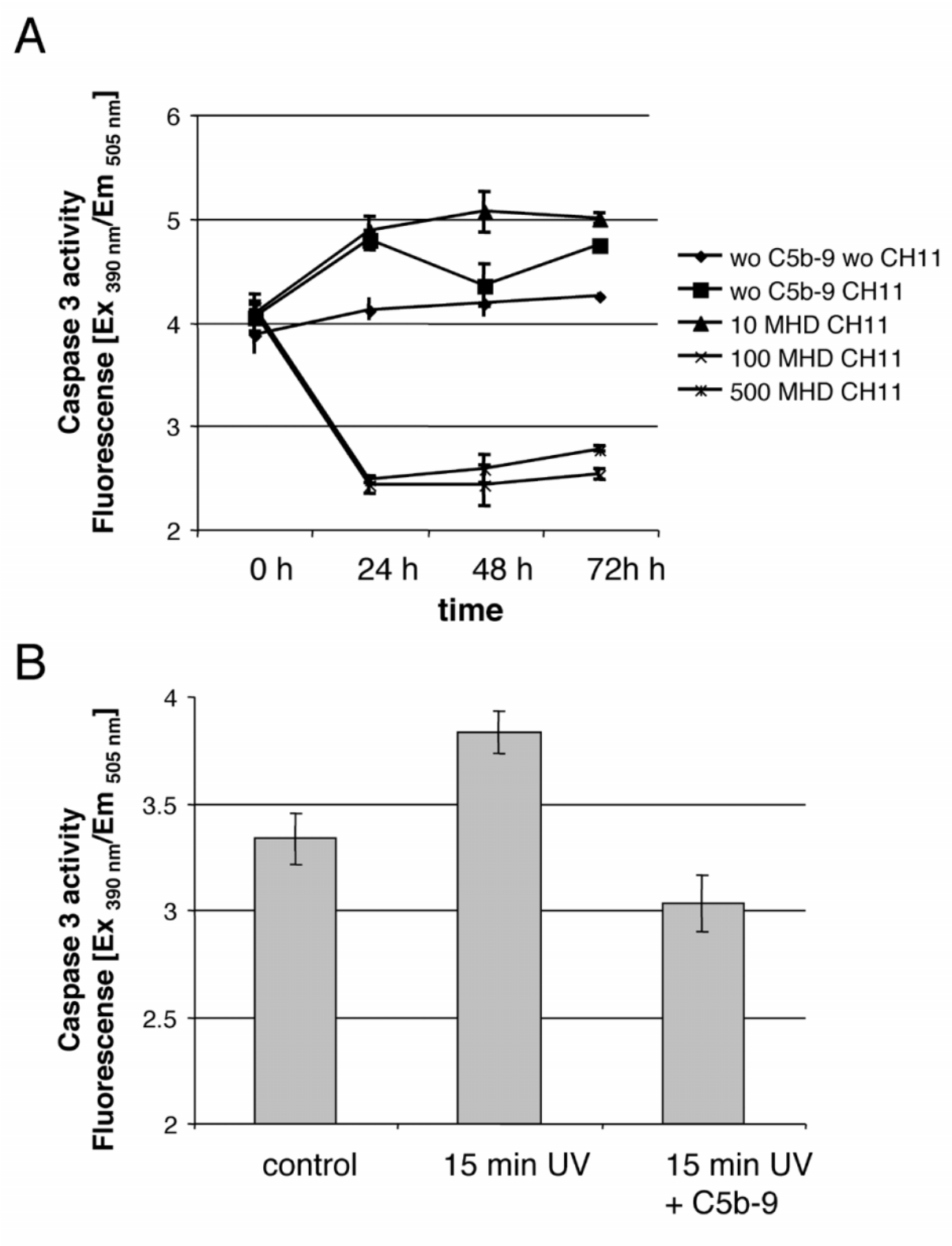

Figure 2. Caspase-3 activity assays. Time-dependent inhibition of caspase-3 activity following CH11 stimulation of SMCs preincubated with 100 MHD C5b-9 ($P<0.05$) and 500 MHD C5b-9 ($P<0.05$). **A)** 10 MHD sublytic C5b-9 activity had no antiapoptotic effect. **B)** Caspase-3 activity was also reduced after ($P<0.05$) UV light treatment of C5b-9-pretreated cells.

Fig. 3

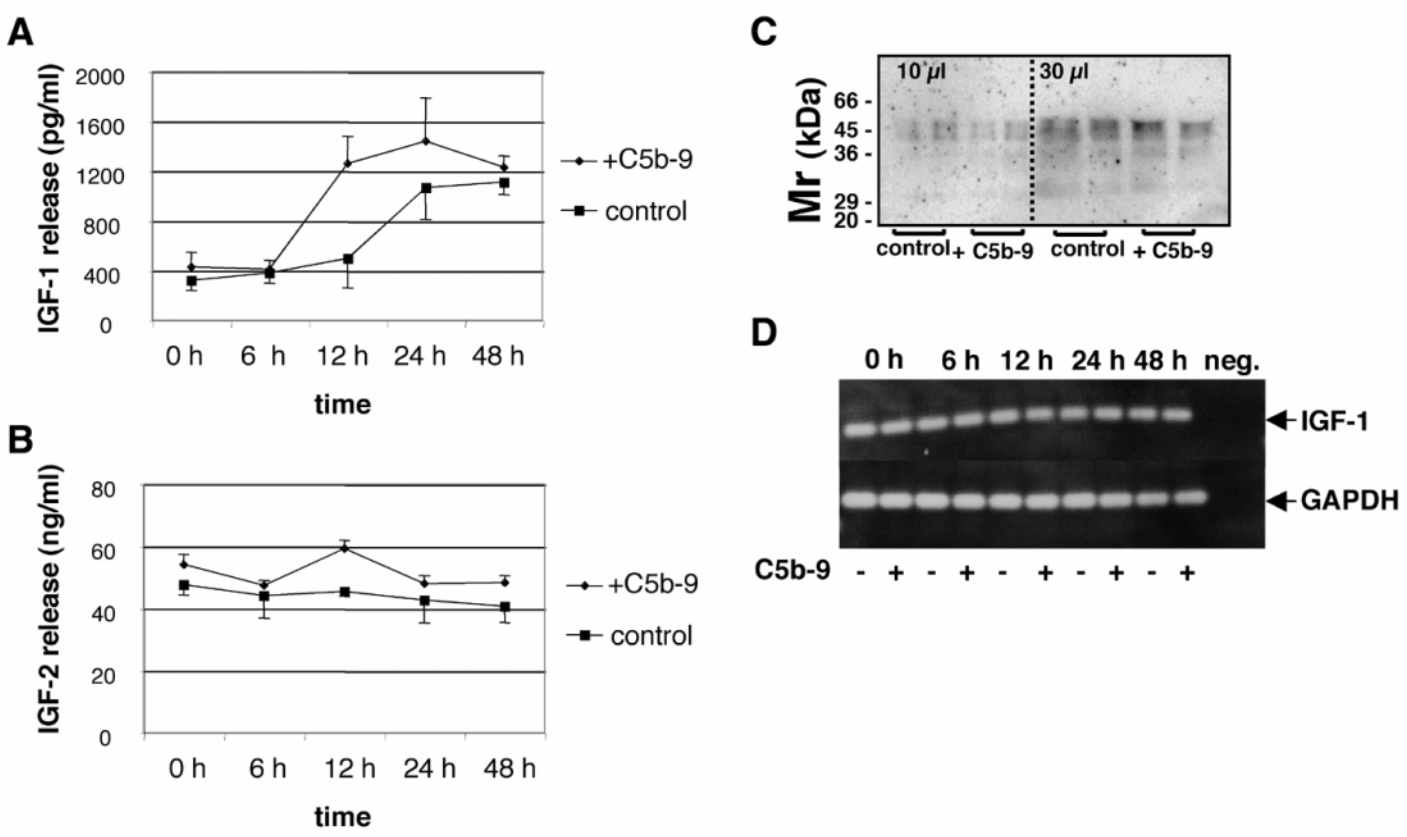

Figure 3. Secretion of IGF system components. As determined by RIA, C5b-9 significantly increases IGF-1 release ($P<0.01$) from SMCs in a time-dependent manner, with maximum amount after 24 h (**A**), whereas IGF-2 secretion is not influenced (**B**) ($n=4$). **C)** Western ligand blot analysis of conditioned media after 18 h revealed the secretion of different IGFBPs with apparent molecular weights of 24, 32, and 36–45 kDa. IGFBP secretion does not differ after stimulation with C5b-9 (Western ligand blot loading volumes as indicated). **D)** RT-PCR with IGF-1-specific primers revealed no difference in mRNA expression between cells preincubated with sublytic C5b-9 and control cells.

Fig. 4

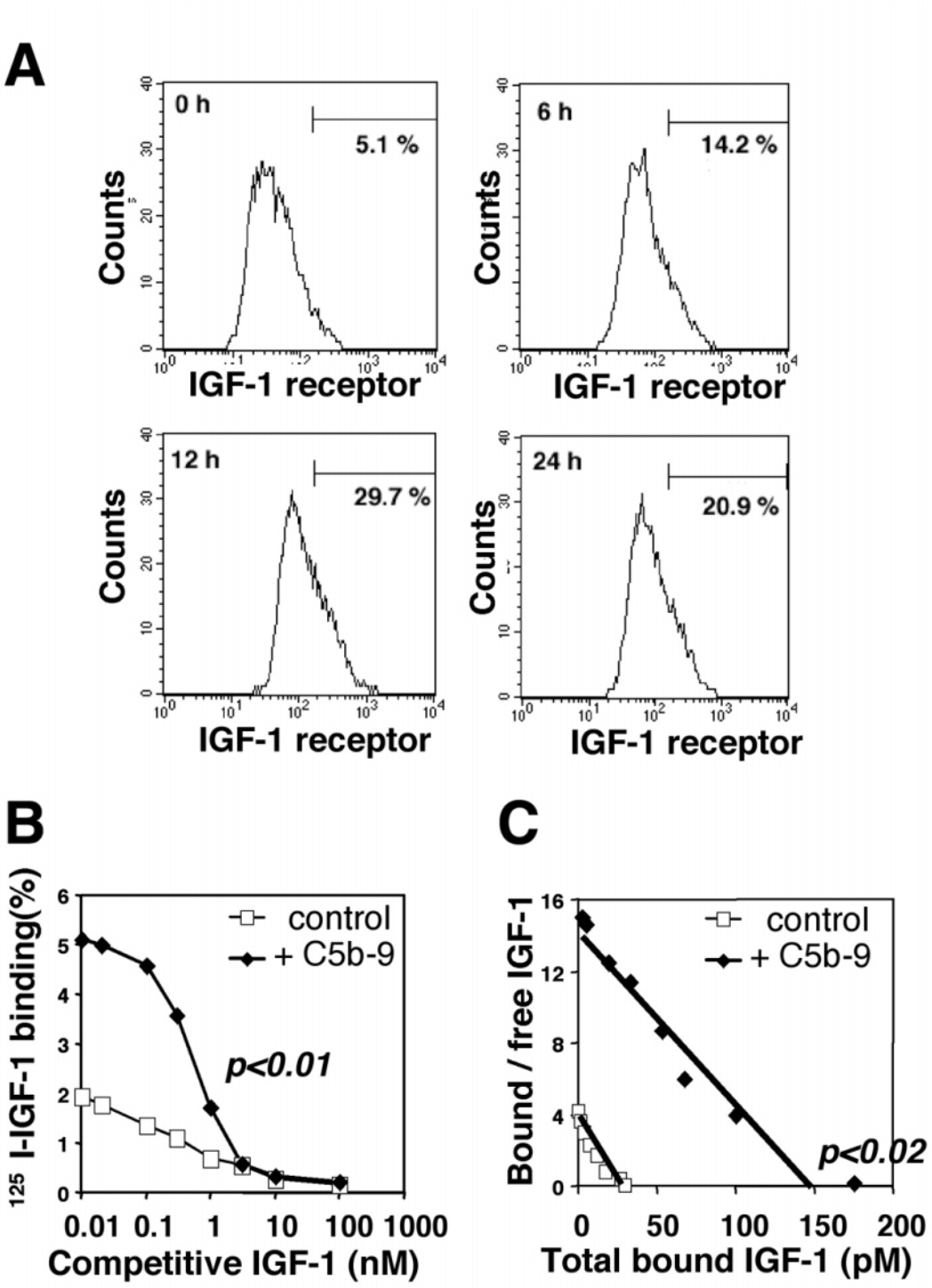

Figure 4. IGF-1 receptor binding. A) Flow cytometric analysis revealed a time-dependent up-regulation of IGF-1 receptors following sublytic C5b-9 stimulation. **B)** As determined by ligand binding assays, incubation with sublytic C5b-9 for 18 h induces a significant increase (P<0.01) in the amount of specifically bound ^{125}I-IGF-1. **C)** Scatchard analysis revealed an increase (P<0.02) in receptor number without changes in binding affinity. Mean values of four separate binding assays are shown.

Fig. 5

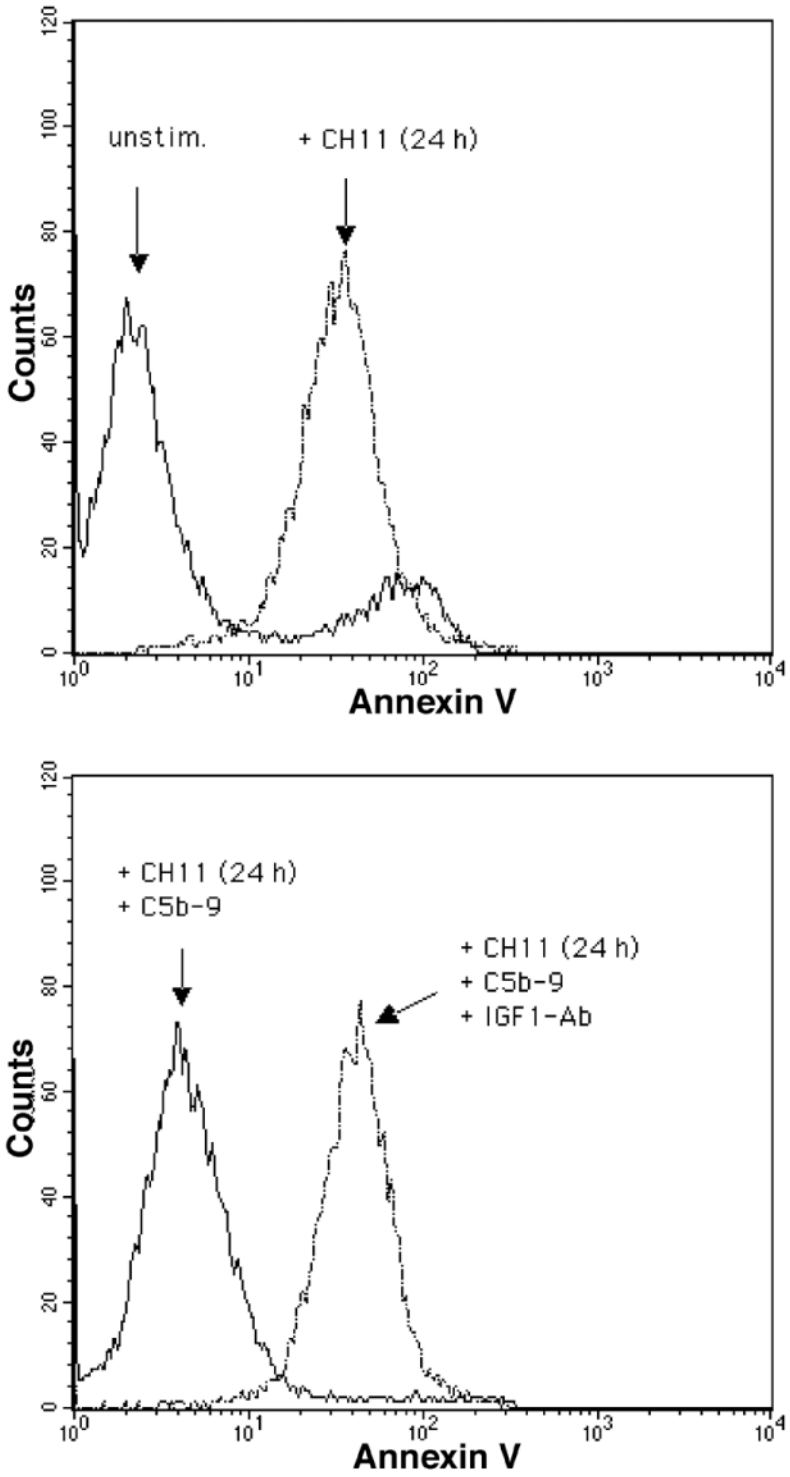

Figure 5. Involvement of IGF-1 in antiapoptotic properties of C5b-9. SMCs were stimulated with sublytic C5b-9 and incubated with a mouse anti-human CD95 monoclonal antibody (CH11) or control isotype-matched antibodies (unstimulated) for 24 h. To assess whether autocrine-released IGF-1 may be involved in C5b-9-mediated inhibition of apoptosis, we preincubated SMCs for 24 h with a monoclonal IGF-1 antibody (IGF-1-Ab). Immunoneutralization of endogenously released IGF-1 abolished the antiapoptotic effects of C5b-9.

Fig. 6

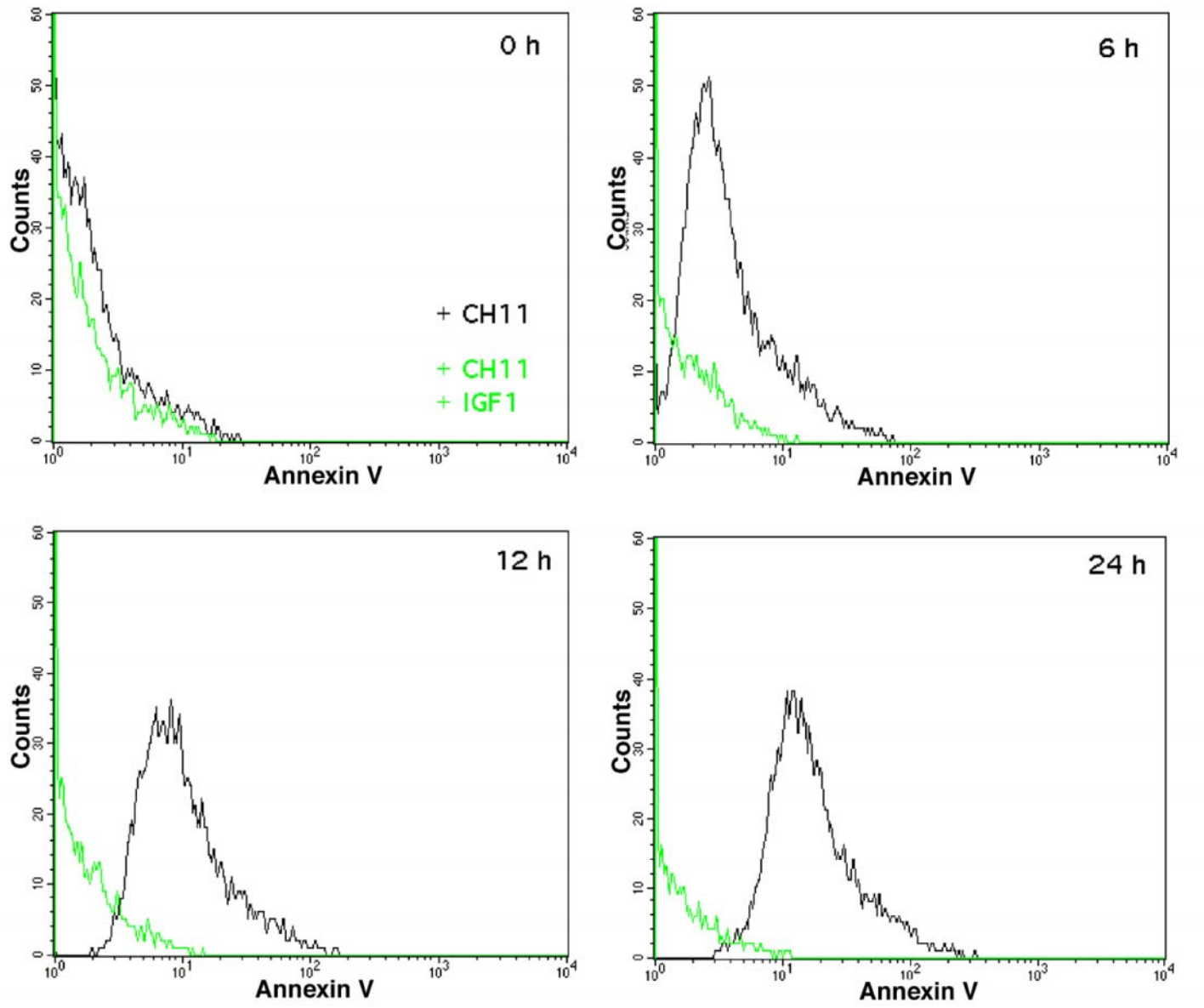

Figure 6. IGF-1 is antiapoptotic for SMCs. Time-dependent enhancement of annexin V binding following CH11 stimulation of SMCs (black). Preincubation of SMCs with recombinant IGF-1 (green) inhibits shift in annexin V-binding.