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Expression of Cellular FLICE-Inhibitory Protein in Human Coronary Arteries and in a Rat Vascular Injury Model

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We previously isolated MACH-related inducer of toxicity (MRIT), a homolog of caspase 8. MRIT, also known as c-FLICE-inhibitory protein (c-FLIP), is an enzymatically inactive homolog of caspase 8 with homology to viral FLIP (v-FLIP). Because of this homology and resemblance to dominant negative proteins, c-FLIP is widely believed to be an antagonist to the death receptor-initiated apoptotic pathways that use caspase 8. We generated a polyclonal antibody, MAG1, and show that this antibody specifically recognizes two splice forms, long form (c-FLIPL) and short form (c-FLIPS). By *in situ* hybridization and immunohistochemistry, we demonstrate that c-FLIP is expressed in endothelial cells, macrophages, and smooth muscle cells (SMCs) both in human coronary arteries and in cultured cells. In an uninjured rat carotid arteries, c-FLIP protein is abundant in the vascular media. After balloon angioplasty, c-FLIP protein is rapidly down-regulated in medial SMCs for 2 weeks and regains expression by 4 weeks. In contrast, the neointima is strongly immunoreactive to c-FLIP from day 7 after the initial injury and remains strongly immunoreactive until 4 to 6 weeks. Similarly there is strong c-FLIP immunoreactivity in SMCs from nonatherosclerotic diffuse intimal thickening and in the overlying endothelial cells. In contrast, c-FLIP immunoreactivity is uneven and often absent in SMCs within the atherosclerotic plaque. Double labeling with c-FLIP antibody and terminal deoxynucleotidyltransferase-mediated UDP end labeling (TUNEL) in the injured rat common carotid artery show that TUNEL-positive cells in the first 2 days after injury lack detectable c-FLIP, suggested a role for caspase 8 in this form of death. In contrast, there is no correlation of c-FLIP with the spontaneous elevation in death of intima seen at 7 days after injury. For human atherosclerotic plaques, the majority of TUNEL-positive cells lack detectable c-FLIP. The expression pattern of c-FLIP and the relation between c-FLIP and TUNEL suggest a role for c-FLIP- and caspase 8-driven death in control of viability of the cells of the atherosclerotic intima. ***(Am J Pathol 2000, 156:125–137)***

Studies from several laboratories have suggested that apoptosis may play an essential role in atherogenesis and vascular remodeling.[1–3] Understanding how apoptosis is regulated in the nematode (Caenorhabditis *elegans*) has provided a molecular framework for cell death. In *C. elegans*, cell death or survival is dependent on a balance between pro- and antiapoptotic homologs of Ced-9 and Egl-1.[4,5] However, regulation in higher organisms is more complex, and at least 15 homologs of these BCL proteins have been described in mammalian cell systems.[6] In addition to the regulators of the Bcl-2 family, the mammalian cell death pathway is more complex because the prototypic Ced-3 protease has evolved into a protease cascade that amplifies death signals initiated by multiple signaling pathways including the Fas/tumor necrosis factor-α receptor 2 (TNF-αR2) "death" receptor family.[7] Transmission of a death signal via this cascade, even when the receptors are ligated, depends on endogenous regulators of proteolytic activation. For example, viral Fas-associated death domain-like IL-1β-converting enzyme (FLICE) inhibitory protein (v-FLIP), an anti-apoptotic viral caspase antagonist, has homology to the death effector domains (DEDs) in the prodomains of caspases 8 and 10.[8] Like DED constructs of caspase 8, the viral FLIPs act as dominant negatives for Fas-associating protein with death domain (FADD)-mediated death, apparently by competitive binding of the predomains of caspase 8 or 10 and thus the blocking of Fas-mediated apoptosis.[8]

Recently, we and others[9–16] have identified a mammalian molecule that is homologous to caspase 8. We named this molecule MACH-related inducer of toxicity (MRIT)[13]; however, it has also been called cellular FLIP (c-FLIP),[9] Casper,[10] FADD-like antiaptotic molecule 1 (FLAME-1),[11] caspase homolog (CASH),[12] I-FLICE,[14]

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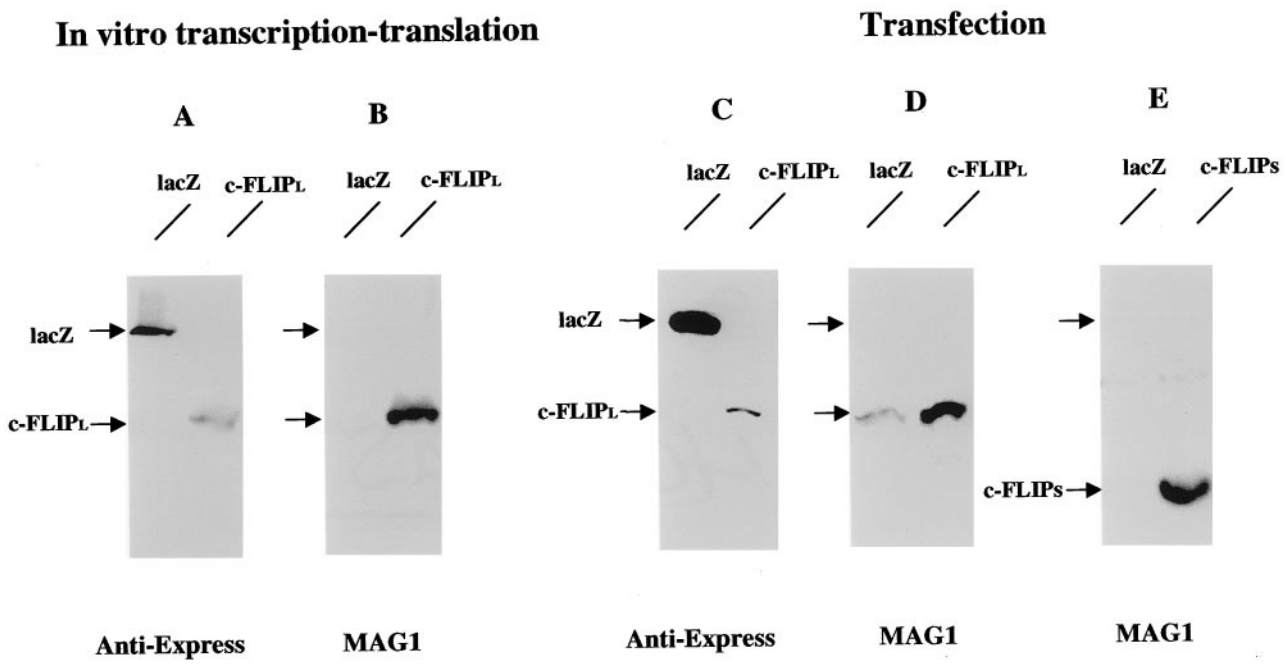

Figure 1. Specifc immunoreactivity of a polyclonal antibody (MAG1) to c-FLIP proteins. Protein expression of epitope-tagged c-FLIPL (long form) and c-FLIPs (short form) by coupled *in vitro* transcription-translation (**A**, **B**) or transient transfection in mammalian cells (**C**, **D**, **E**). An epitope-tagged β-galactosidase gene product (LacZ) was used as a control for protein expression. Western analyses with an anti-express epitope antibody (**A**, **C**) and MAG1 antibody (**B**, **D**, **E**) are shown. A weak immunoreactive band at the LacZ lane in panel D represents endogenous c-FLIPL.

CLARP,[15] and usurpin.[16] For simplicity, we will use the term c-FLIP in this paper. c-FLIP is different from v-FLIP in containing a full-length sequence that includes, although in a distinct form, the active site of caspase 8. Interestingly, c-FLIP is very similar in structure to a dominant negative construct of caspase 8,[9–16] and this has led to the presumption that c-FLIP is also an antiapoptotic molecule. In support of this hypothesis, studies in T cells show that c-FLIP levels are controlled during activation and go down when activated T cells become susceptible to Fas-mediated death,[9] and the ability of Fas to kill T cells can be blocked if c-FLIP levels are restored using a chimeric form of c-FLIP ligated to viral membrane transport protein.[17] Thus, although early studies, including our own,[13] have shown that overexpressed c-FLIP induces cell death, the preponderance of the evidence is that this molecule is a natural inhibitor of death, mediated by those death receptors that are able to activate caspase 8 by Fas signaling or by other receptors. Fas is ubiquitously expressed in various tissues including the vessel wall.[18,19] To explore possible functions of c-FLIP in atherosclerosis and vascular responses to injury, we have used *in situ* hybridization and immunohistochemistry to analyze the pattern of c-FLIP expression in human coronary arteries and in injured rat common carotid arteries. We find that c-FLIP is widely expressed in the normal vessel wall. c-FLIP is, moreover, up-regulated in rat neointimal lesions, as well as in the intima of nonatherosclerotic human coronary arteries. Double labeling for c-FLIP and terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) in the injured rat common carotid artery shows that loss of c-FLIP in the first few days after injury is correlated with the appearance of TUNEL-positive cells. For human atherosclerotic plaques, the majority of TUNEL-positive cells lack detectable c-FLIP.

Materials and Methods

Tissue Collection, Preparation, and Characterization

Human coronary arteries were obtained from hearts removed from patients with end-stage ischemic cardiomyopathy (n = 6), idiopathic dilated cardiomyopathy (n = 5), or congenital heart disease (n = 2), a total of 9 men and 4 women, 26 to 63 years of age (see Table 2 below). Twenty-one coronary artery segments were obtained from the hearts of these 13 subjects. These segments were classified into two groups by classic histological criteria: one comprising artery segments that demonstrated nonatherosclerotic diffuse intimal thickening (DIT; n = 9) and the other comprising segments that demonstrated atherosclerotic plaques (n = 12). Collection and use of these tissues were approved by the University of Washington Human Subjects Review Committee.

Rat Balloon Injury Models

The rat balloon injury model was described previously.[20] Briefly, 3-month male Sprague-Dawley rats (around 450 g; Zivic Miller, Zelienople, PA) were used for these studies (n = 6 for each time point). All animals were given rat chow and water *ad libitum.* The animals were anesthetized with ketamine HCl (50 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg) administered intramuscu-

larly in the back. Osmotic minipumps containing bromodeoxy uridine (BrdU; 30 mg/ml) were implanted to measure DNA synthesis as described previously.[20] The left common carotid artery was de-endothelialized by passage of a 2F Fogarty embolectomy catheter (Edwards Laboratories, Santa Ana, CA) that was inserted into the external carotid artery, advanced into the abdominal aorta, inflated, and withdrawn. This procedure was performed three times to ensure complete endothelial denudation of the common carotid artery. The external carotid was tied off, and blood flow was restored through the internal carotid artery. For morphological analysis and protein extraction, the rats were sacrificed at 2, 7, 14, 28, or 42 days after the injury by an overdose injection of sodium pentobarbital (intravenous Nembutal, Abbott Laboratories, North Chicago, IL). The animals were prepared by perfusion/fixation using 4% paraformaldehyde as described previously.[20] Ten minutes before death by pentobarbital overdose, these rats received an intravenous injection of Evans blue (200 μL of 5% solution; Sigma Chemical Co., St. Louis, MO) to mark the de-endothelialized area. The carotid arteries were briefly flushed with ice-cold lactated Ringer's solution (Baxter Healthcare Co.) at physiological pressure to remove blood, and the carotid arteries were excised. The adventitia was stripped (at the external elastic lamina) from the media. All specimens then were snap-frozen in liquid nitrogen and stored at −80°C. The Animal Care Committee of the University of Washington approved all procedures.

Cell Culture

Culture of coronary plaque smooth muscle cells (SMCs), normal aortic and neonatal medial SMCs, human umbilical endothelial cells and human microvascular endothelial cell-1 cells has been described previously.[21] SMCs were grown in Waymouth's media supplemented with 20% fetal bovine serum (FBS) together with 1× antimycotic and antibiotic agents.

c-FLIP Antibody Preparation and Characterization

A peptide of the N-terminal DED of c-FLIP corresponding to amino acids 10 to 27 (sequence EEALDTDEKEMLIFL-CRD) was used to generate an affinity-purified rabbit polyclonal antibody, termed MAG1. To examine the c-FLIP specificity of MAG1, we used an *in vitro* transcription and translation (Promega-TNT) system in conjunction with mammalian cell transfections. Full-length c-FLIP was cloned into the PCDNA 3.1 vector with an anti-express fusion epitope and then translated in vitro followed by Western blotting and detection with either an express antibody (Invitrogen, Carlsbad, CA) or MAG1 (Figure 1, A and B). Baby hamster kidney-21 cells that had been stably transfected with CrmA were transiently transfected with a c-FLIP expression vector that encodes c-FLIPL and c-FLIPS, using Fugene 6 (Roche Biochemicals, Indianapolis, IN) or Lipofectamine (GIBCO/BRL, Rockville, MD) as described previously[13] (see Figure 1).

Immunohistochemistry and TUNEL

The primary antibodies used for immunohistochemical analyses include a polyclonal antibody against c-FLIP (MAG1, 1:100 dilution), a monoclonal antibody (mAb) recognizing endothelium (CD31; DAKO, Carpinteria, CA; 1:1000), an mAb-recognizing macrophage (CD68; DAKO; 1:1000), and an mAb recognizing SMC α-actin (Boehringer Mannheim, Indianapolis, IN; 1:2000). All antibodies were diluted in phosphate-buffered saline (PBS) containing 10% normal goat serum and 1% bovine serum albumin and incubated with the tissue sections for 1 hour at room temperature. Negative controls included substitution of the primary antibody with nonimmune immunoglobulin G (IgG) of the same class. In addition, competitive inhibition of the MAG1 antibody with the immunizing peptide was performed by preincubating the two for 30 minutes to 1 hour at 37°C. All immunohistochemical- and TUNEL-stained sections were scored by two observers who were blinded for the specimen identity and the antibody used. The method for TUNEL with alkaline phosphatase has been described previously.[1] TUNEL analyses were always performed, including human tonsil and rat thymus as positive controls of human and rat samples, respectively, to provide internal consistency. Cell proliferation was measured by BrdU infusion and immunohistochemical detection as previously reported.[20] The TUNEL-labeling and -proliferating cells for each high-power field were shown as a percentage of total cells (i.e., TUNEL- or BrdU-labeling index = TUNEL- or BrdU-positive cells/total cells per high-power field × 100 (n = 6) injured rat common carotid arteries per time point). Nonspecific cytoplasmic TUNEL staining without nuclear involvement was considered negative. For the human coronary arteries (n = 21), both the TUNEL-labeling index and c-FLIP intensity in the intimal region, medial region, and endothelium were simultaneously examined on double-stained slides. At least four sections from each specimen were examined. Ten random fields per section were examined at high magnification (×100). A total of 200 to 400 cells from each section were counted at high power (×100). The results of immunohistochemical studies for c-FLIP expression were assessed by scoring the intensity for a given antigenic determinant, using a scale of 0 = negative, 1+ = weak, 2+ = moderate, and 3+ = strong. The median was then assigned for all individual scores.

Double Staining

The first immunostaining using MAG1 antibody was developed with 3,3′-diaminobenzidine (Sigma), which produced a brown reaction product. The second sequence of staining was done on the same sections for TUNEL, with an avidin-alkaline phosphatase-substrate system and Vector Red (Vector ABC Kit, Vector Laboratories), which produced a red reaction product.

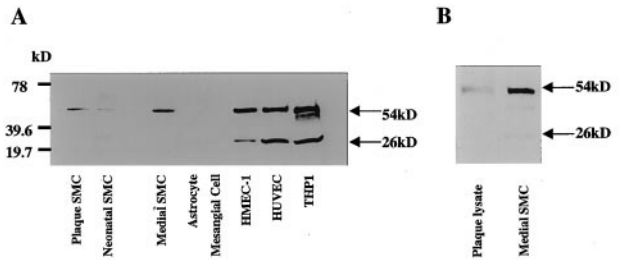

Figure 2. Expression of endogenous c-FLIP proteins. **A:** Western analyses (30 μg total cell lysates/lane), using MAG1 antibody detected c-FLIP$_L$ protein in human VSMCs (**lanes 1–3**), endothelial cells (**lanes 6**, **7**), and THP1 cells (**lane 8**). A shorter form, representing c-FLIPs, is also seen in endothelial cells (**lanes 6**, **7**) and in THP1 (**lane 8**). Astrocytes and mesangial cells express very low or undetectable levels of c-FLIP$_L$ or c-FLIPs. **Lanes: 1**, plaque SMC; **2**, neonatal SMC; **3**, medial SMC; **4**, astrocyte; **5**, mesangial cells; **6**, human microvascular endothelial cells (HMEC-1); **7**, human umbilical endothelial cells (HUVECs); **8**, monocytic cell line THP1. **B:** A low level of c-FLIP$_L$ expression in human atherosclerotic *in vivo* plaque lysate (**lane 1**, 30 μg total cell lysates/lane) compared with normal medial SMCs (**lane 2**, 30 μg total cell lysates/lane).

Preparation of Riboprobes

Full-length human c-FLIP complementary DNA (cDNA) (1.4 kb) was subcloned into pBluescript II (Stratagene, La Jolla, CA) as described previously.[13] To generate antisense and sense RNA probes for *in situ* hybridization, plasmids were linearized with XbaI and KpnI, respectively. The plasmids were then *in vitro* transcribed with ^{35}S-labeled α-thio UTP (New England Nuclear-Dupont, Boston, MA) by a modified method of Wilcox et al.[22] To improve penetration into sections during hybridization, transcripts were shortened by alkaline hydrolysis to a calculated average length of 250 bases.

In Situ *Hybridization*

In situ hybridizations was performed with ^{35}S-labeled riboprobes on 5-μm-thick, deparaffinized sections of 4% paraformaldehyde-fixed tissue. Riboprobes were separated from unincorporated counts by passage over G-50 NICK columns (Pharmacia, Piscataway, NJ). The peak radioactive fractions were treated with phenol/chloroform, and the aqueous phase was precipitated with ethanol and resuspended to 300,000 cpm/ml in TE buffer (10 mmol/L Tris, 1 mmol/L ethylenediaminetetraacetic acid, pH 7.5). One microliter of the riboprobe was mixed in 50 μl hybridization buffer and applied to each section, and hybridizations were performed at 55°C overnight. Washes included treatment with RNase A (20 mg/ml, Sigma) for 30 minutes at 37°C and a stringent wash in 0.1 × standard saline solution at 55°C for 2 hours. After dehydration in graded alchols and air-drying, slides were dipped in NTB2 emulsion (Eastman Kodak Co., Rochester, NY), exposed in the dark at 4°C for 14 days, and developed as described previously.[22] Hematoxylin was used as a counterstain.

Western Blotting

c-FLIP protein was detected by using whole-cell lysates and tissues including human vascular plaque and uninjured or injured rat common carotid arteries at several time points. The samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to poly(vinylidene difluoride) membranes, and processed as described previously.[23] The protein concentration of the lysate was determined using the Pierce micro BCA reagents (Rockland, IL). The MAG1 antibody was used at a dilution of 1:100 and a final concentration of 1.4 μg/ml of peptide affinity-purified IgG. The secondary antibody, an anti-rabbit IgG labeled with horseradish peroxidase, was used at a dilution of 1:30,000 (Biorad Laboratories, Hercules, CA). The signals was visualized by enhanced chemiluminescence (ECL; Amersham). Intensity of the signal was measured by densitometric analysis of autoradiograms. X-ray films were scanned with a transmission scanner (UMAX UC1260, UMAX Data System Inc.) using Adobe Photoshop software (version 3.0; Adobe), and the transmission values were converted to values of optimal density by NIH Image software (version 1.59; National Institutes of Health, Bethesda, MD). The profile of each band was plotted by using NIH Image, and the densitometric intensity corresponding to each band was measured as an intensity value. c-FLIP protein intensity was normalized by using calponin expression.

Results

c-FLIP Expression in Cultured Endothelial Cells, SMCs, and Macrophages

Although a number of antibodies to c-FLIP are available commercially, we wished to be sure of the specificity of our MAG1. Expression data and protein data show that the gene can be transcribed as at least two forms; c-FLIPs contains only the regions homologous with the viral protein and c-FLIP$_L$ is a full-length transcript.[24] We first tested the specificity of the MAG1 antibody. This antibody is expected to recognize c-FLIP$_L$ and c-FLIPs proteins, because these isoforms are identical in their amino acid composition at the N-terminal death effector domain (DED).[13] Using both *in vitro* transcription-translation and transfection analyses, we found that the MAG1 antibody specifically recognized a 54-kD protein (Figure 1, B and D) but not the LacZ gene product (Figure 1, A and C), representing the correct size of c-FLIP$_L$ in both assays. As a positive control for protein expression, we used express epitope-tagged LacZ and c-FLIP constructs in both assays. As shown in Figure 1 (A and C), both LacZ and c-FLIP proteins were detected by antiexpress antibody. The MAG1 antibody also recognized c-FLIPs in transfected mammalian cells (Figure 1E).

We next used the MAG1 antibody to test the expression of endogenous c-FLIP in total cell lysates from different SMCs, endothelial cells, and THP1, a monocytic cell line. As shown in Figure 2A, a 54-kD c-FLIP$_L$ protein was detected by MAG1 antibody in cultured plaque SMCs (lane 1), neonatal VSMCs (lane 2), medial VSMCs (lane 3), human microvascular endothelial cell-1 (lane 6), human umbilical endothelial cells (lane 7), and THP1

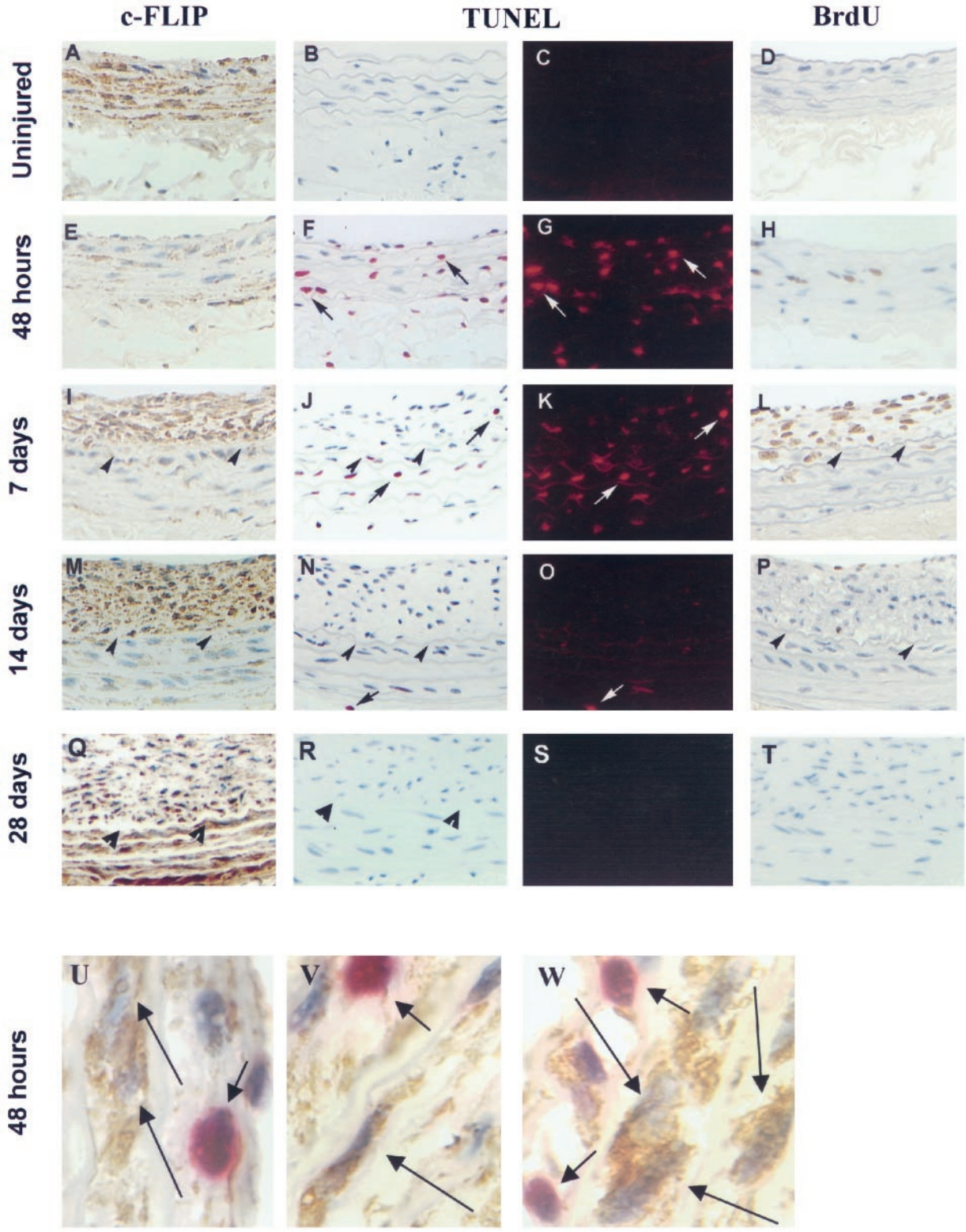

Figure 3. High levels of c-FLIP expression in the neointima and injury-mediated down-regulation of c-FLIP in the media partially coincide with TUNEL positivity. c-FLIP is abundant in the cytoplasm of medial SMCs of the uninjured vessel and greatly reduced or absent in the same cells at 48 h after injury. Time-course expression of c-FLIP, TUNEL, and BrdU incorporation in uninjured rat carotid arteries (**A–D**), and in injured rat carotid arteries 48 hours (**E–H**), 7 days (**I–L**), 14 days (**M–P**), and 28 days (**Q–T**) after balloon injury is shown. Serial sections were immunostained for c-FLIP (**A**, **E**, **I**, **M**, and **Q**), assayed for DNA fragmentation by TUNEL (**B**, **C**, **F**, **G**, **J**, **K**, **N**, **O**, **R**, and **S**), or immunostained for BrdU incorporation (**D**, **H**, **L**, and **P**). Because TUNEL was developed with a red dye that is also fluorescent, Vector Red, detection of TUNEL-positive cells from the carotid arteries (**B**, **F**, **J**, and **N**) were also examined under a fluorescence microscope (**C**, **G**, **K**, and **O**). Arrows indicate TUNEL-positive cells. **Arrowheads** indicate internal elastic laminae. Note that although the down-regulation of c-FLIP correlates with TUNEL positivity in the medial layer at 48 hours (**E**, **F**) and 7 days (**I**, **J**), the majority of the medial SMCs that contained low levels of c-FLIP are TUNEL-negative at day 14 (**M**, **N**). Double labeling of carotid arteries with TUNEL and c-FLIP 48 hours after balloon injury demonstrates that TUNEL-positive cells (red) are c-FLIP negative (**U**, **V**, **W**, **short arrows**), and c-FLIP positive cells (brown) are TUNEL-negative (**U**, **V**, **W**, **long arrows**). Six animals for each time point were examined, and a representative section for each experiment is shown. Original magnifications: **A–T**, ×40; **U–W**, ×100.

Table 1. Relation between c-FLIP Expression and TUNEL Positivity in the Media and Intima of Injured Rat Common Carotid Arteries

Days after injury	Localization	TUNEL(+)		TUNEL(−)	
		c-FLIP (+)	c-FLIP (−)	c-FLIP (+)	c-FLIP (−)
2	Media	0.8 ± 0.5	39.8 ± 2.7	23.5 ± 2.2	35.8 ± 3.4
7	Media	1.3 ± 0.6	38.2 ± 1.2	20.5 ± 1.3	40.0 ± 1.1
	Intima	24.3 ± 1.0	4.0 ± 0.6	68.7 ± 1.1	3.0 ± 0.7
14	Media	0 ± 0	5.0 ± 1.5	14.2 ± 2.1	80.8 ± 2.8
	Intima	0 ± 0	4.3 ± 1.2	94.5 ± 1.3	1.2 ± 0.7

Rat balloon-injured arteries were stained by immunostaining with MAG1 antibody against c-FLIP, followed by TUNEL; $n = 6$ sections from animals at each time point. Six random fields per section were examined at high magnification (×100). Approximately 100 cells were counted per section for TUNEL and c-FLIP single and double positive and/or negative. Data represent means ± SEMs.

(lane 8). On the other hand, c-FLIP protein was not detectable in astrocyte and mesangial cells (lanes 4 and 5, respectively). Additionally, 54-kD c-FLIP$_L$ protein was also detectable in human coronary plaque lysates (Figure 2B).

In Vivo *Arterial Injury*

We next examined c-FLIP expression in a rat vascular balloon injury model. In uninjured rat carotid arteries, strong immunoreactivity to c-FLIP protein was detected in the medial SMCs (Figure 3A). However, 48 hours after balloon injury, c-FLIP immunoreactivity was greatly diminished (Figure 3E). At this time point, TUNEL-positive cells were abundant in the media (Figure 3, F and G). Detailed analysis by high-power magnification of carotid arteries from the 48-hour time point doubly stained for c-FLIP and TUNEL revealed that TUNEL-positive cells lacked c-FLIP protein (Figure 3, U–W, short arrows) and c-FLIP-positive cells (Figure 3, U–W, long arrows) also lacked TUNEL positivity. The counts at 48 hours showed that only 0.8% of cells with c-FLIP were TUNEL positive in comparison with 39.8% of cells without c-FLIP (Table 1). Medial SMCs at day 7 stained weakly for c-FLIP, and high levels of TUNEL-positive cells could be detected in the media at this stage (Figure 3, I–K; Figure 4B). By day 14, medial SMCs remained to stain weakly for c-FLIP, although the frequency of TUNEL positivity is low. When c-FLIP expression was regained in the media by week 4, no TUNEL-positive cells were found (Figure 3, Q–S). Interestingly, neointima was strongly immunoreactive to c-FLIP at day 7 after the injury and remained strongly immunoreactive until 4 to 6 weeks (Figure 3Q; results not shown). On day 7, a number of TUNEL-positive cells could be detected in the intima, and 24.3% TUNEL-positive cells were also found to be c-FLIP positive (Figure 3, I–K; Table 1). However, by day 14 the majority of c-FLIP-positive cells in the neointima were no longer TUNEL positive (Figure 3, N and Q; Table 1). Overall, double staining for c-FLIP and TUNEL in the injured rat common carotid artery suggested a complex regulation of c-FLIP in TUNEL-positive and -negative cells. In addition, c-FLIP expression is down-regulated initially in the media but returns to normal levels by 28 days after the injury.

To confirm the expression of c-FLIP in rat carotid arteries after balloon injury, we performed Western blot analysis using equal amounts of protein extracts (30 μg/

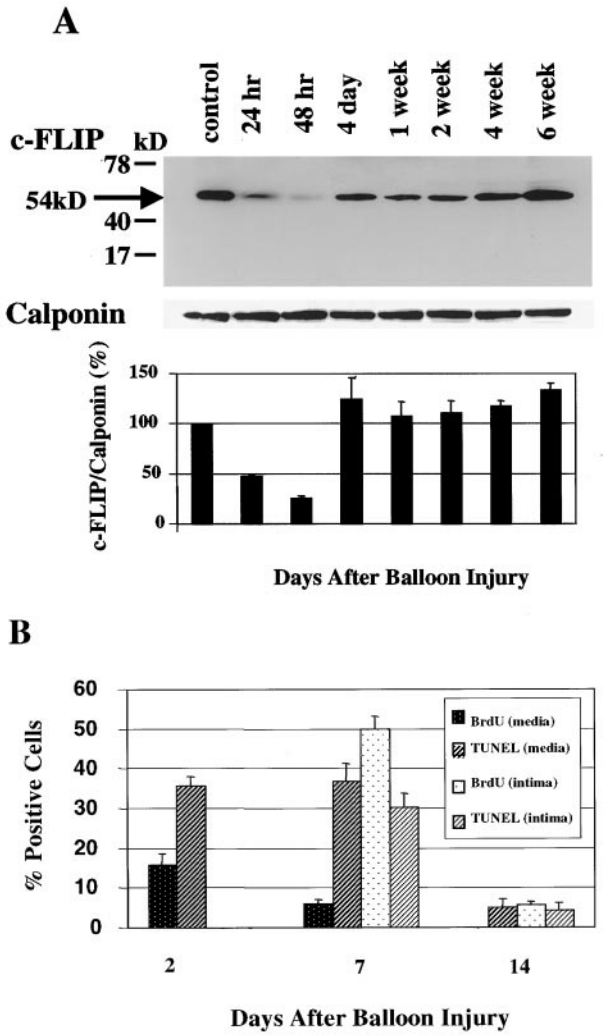

Figure 4. c-FLIP protein is down-regulated at 48 hours and up-regulated by 6 weeks after balloon injury. A representative Western blot analysis of protein lysates (30 μg/lane) from rat carotid arteries after balloon injury at indicated time points (**A**, **top panel**) and quantification by densitometry (**A**, **lower panel**) are shown. Bar graphs show c-FLIP expression normalized by the intensity of calponin. Data were presented as ratios of the text value to the control uninjured carotid arteries (set at 100%). Note that c-FLIP expression was reduced at 24 and 48 hours after balloon injury and up-regulated by 4 days. **B:** Analysis of rat balloon-injured arteries for apoptosis (TUNEL) and cellular proliferation (BrdU); $n = 6$ sections from animals at each time point. Data are mean ± SEM.

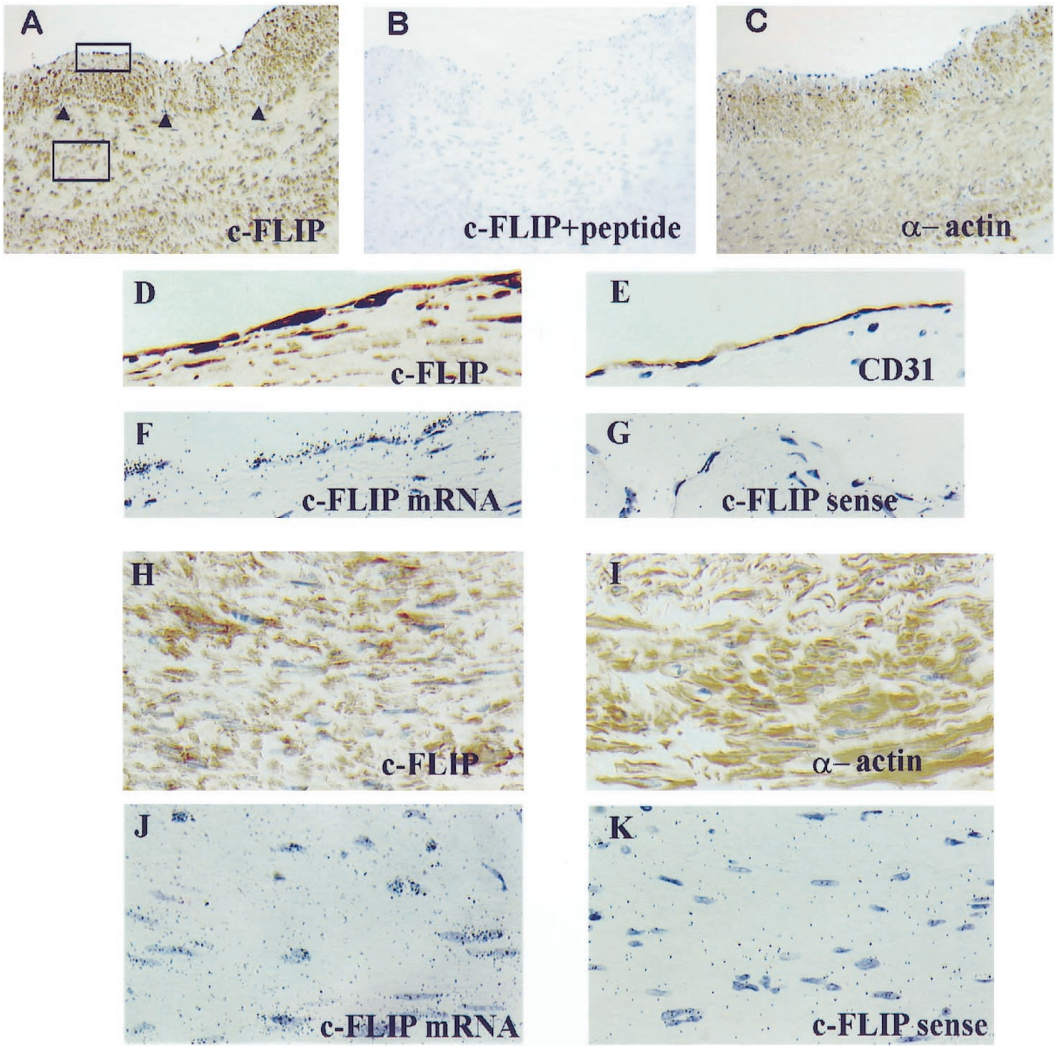

Figure 5. Immunolocalization and *in situ* hybridization of c-FLIP mRNA in a nonatherosclerotic human coronary artery. Low-power photomicrographs of a nonatherosclerotic human coronary artery stained with MAG1 for c-FLIP protein (**A**), MAG1 neutralized with the peptide (**B**), or with anti-α smooth muscle actin antibody (**C**). c-FLIP protein (**A**, **D**, **H**) and mRNA (**F**, **J**) are detected in endothelium (**E**), identified by endothelial cell surface marker CD31 immunohistochemistry, and in vascular SMCs (**I**), identified by smooth muscle α-actin immunohistochemistry. **G** and **K:** Control hybridizations with a sense cRNA probe. A representative sample from a total of nine nonatherosclerotic coronary arteries is shown. Original magnification, **A–C**, ×10; **D–K**, ×40.

lane) from uninjured or injured rat carotid arteries. As shown in Figure 4A, c-FLIP protein was down-regulated at 24 and 48 hours after balloon injury. However, c-FLIP expression was increased at later time points (from day 4 to 6 weeks; Figure 4A). These results are in agreement with immunohistochemical studies of rat carotid arteries after balloon angioplasty (Figure 3). In addition, the results from the Western analysis confirmed that immunoreactivity observed in the vascular cells was due to the expression of a 54-kD cFLIPL protein. c-FLIP protein in the vessel wall after balloon angioplasty was quantified by densitometric analysis of c-FLIP expression from two separate experiments, and band intensities were normalized to calponin expression (Figure 4A). These results suggest that c-FLIP is down-regulated at the 24-h time point after balloon injury and upregulated in the intima and media by 4 weeks.

c-FLIP Expression in Human Coronary Artery and Apoptosis

We next examined the distribution of c-FLIP protein and mRNA in nonatherosclerotic and atherosclerotic human coronary arteries by immunohistochemistry and by *in situ* hybridization. Strong immunoreactivity to c-FLIP protein (also termed DIT) was observed in all nonatherosclerotic lesions, also termed. As shown in Figure 5, strong c-FLIP immunoreactivity was seen in intimal and medial SMCs (Figure 5, A and H) and in endothelial cells (Figure 5, A and D). This immunoreactivity was specific because preincubating the MAG1 antibody with the neutralizing MAG1 peptide completely abolished the immunoreactivity in human coronary arteries (Figure 5B). Immunostaining serial sections from corresponding human coronary arteries with smooth muscle α-actin antibody and CD31

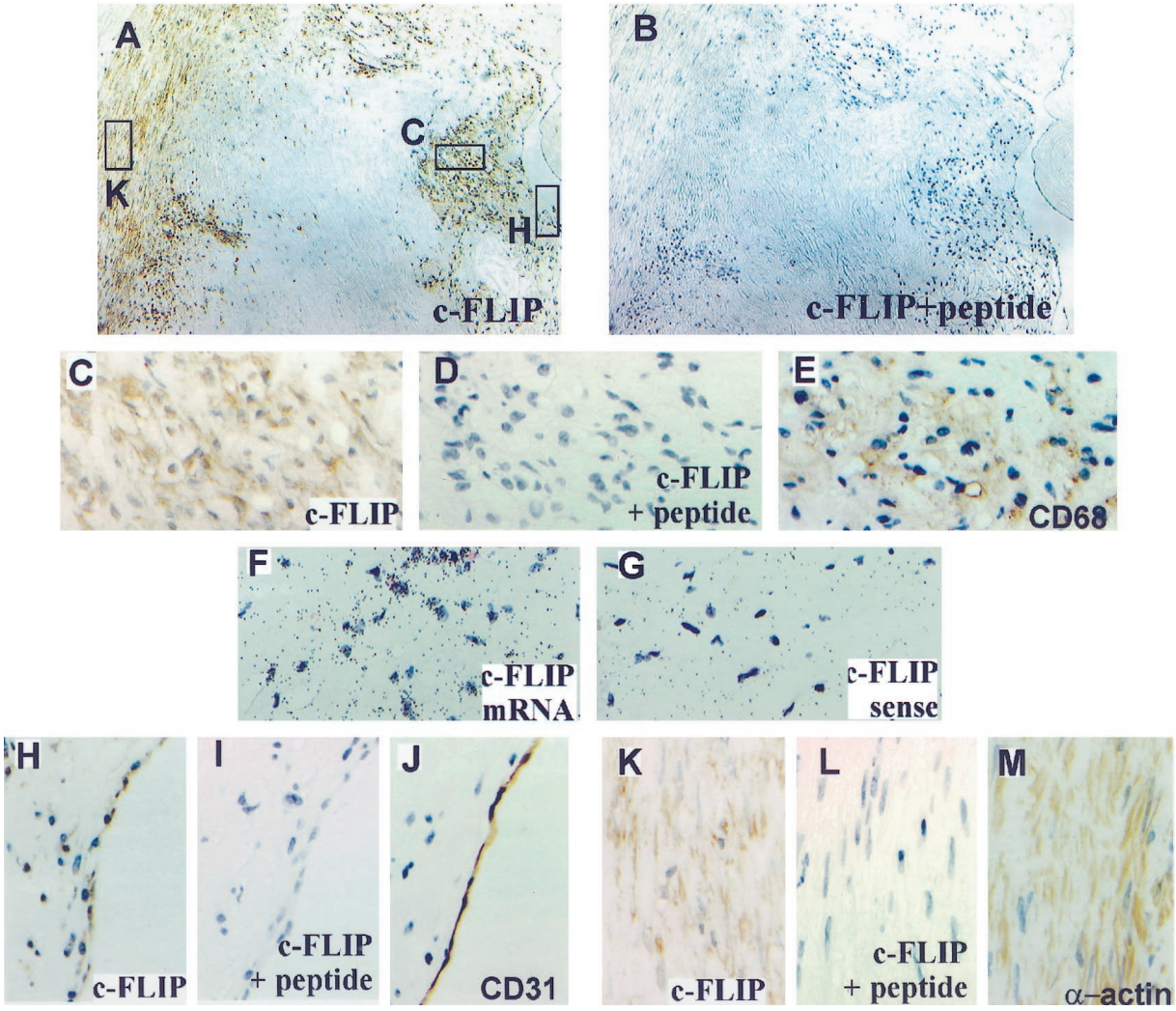

Figure 6. Immunolocalization and *in situ* hybridization of c-FLIP mRNA in a atherosclerotic human coronary artery. **A:** Low-power photomicrograph of an advanced atherosclerotic coronary artery. The inserts outlined distinct areas such as macrophage-enriched area (**C–G**), luminal endothelium (**H**, **J**), and medial smooth muscle layer (**K–M**). c-FLIP protein (**C**) and mRNA (**F**) are detected in macrophages (**C**), identified by macrophage-specific marker CD68 (**E**). **G:** Control hybridization with a sense c-FLIP cRNA probe. Specificity of c-FLIP immunoreactivity was demonstrated by complete neutralization of immunoreactivity by c-FLIP peptide (**B**, **D**, **I**, **L**). Original magnification: **A–B**, ×10; **C–M**, ×40.

antibody revealed that these c-FLIP-positive cells are SMCs and endothelial cells, respectively (Figure 5, C, E and I). Similarly, *in situ* hybridization with an antisense c-FLIP complementary RNA (cRNA; Figure 5, F and J) revealed that c-FLIP messenger RNA (mRNA) was detectable in SMCs and in endothelial cells. No significant signal was detected above the background when the control, sense c-FLIP cRNA, was used to hybridize in nonatherosclerotic coronary arteries (Figure 5, G and K). Interestingly, although c-FLIP was widely expressed in all nonatherosclerotic coronary arteries, TUNEL staining was almost exclusively not detectable in these regions (Table 2).

Next, we examined the expression of c-FLIP in human atherosclerotic coronary arteries. Unlike diffuse staining of the luminal endothelium and SMCs in the nonatherosclerotic samples, two types of c-FLIP staining were seen in atherosclerotic coronary arteries. One type of atherosclerotic plaque showed detectable c-FLIP staining in the luminal endothelium (Figure 6H), medial smooth muscle layer (Figure 6K), and in plaque macrophages (Figure 6, C and F). The second type of atherosclerotic plaque, however, showed weak or undetectable immunoreactivity to c-FLIP in the luminal endothelium (Figure 7C), in the medial smooth muscle layer (Figure 7F), and in the atherosclerotic plaque SMCs (Figure 7I). c-FLIP immunoreactivity was completely abolished when the neutralizing peptide was added in all cases (Figure 6, B, D, I, and L; Figure 7, B, D, G, and J).

The possible correlation between TUNEL and c-FLIP was further tested in a cell-by-cell analysis of human coronary arteries. Double labeling with TUNEL (red reaction product) and c-FLIP (brown reaction product) was performed in 21 human coronary arteries (Table 2). As shown in Figure 8A, TUNEL-positive endothelial cells (arrows) did not express detectable c-FLIP immunoreactivity. Similarly, neither TUNEL-positive macrophages (Figure 8, C and D) nor SMCs (Figure 8, F and G) express detectable c-FLIP. Detailed analysis of human coronary arteries (atheroma and DIT; $n = 21$; Table 2) for possible

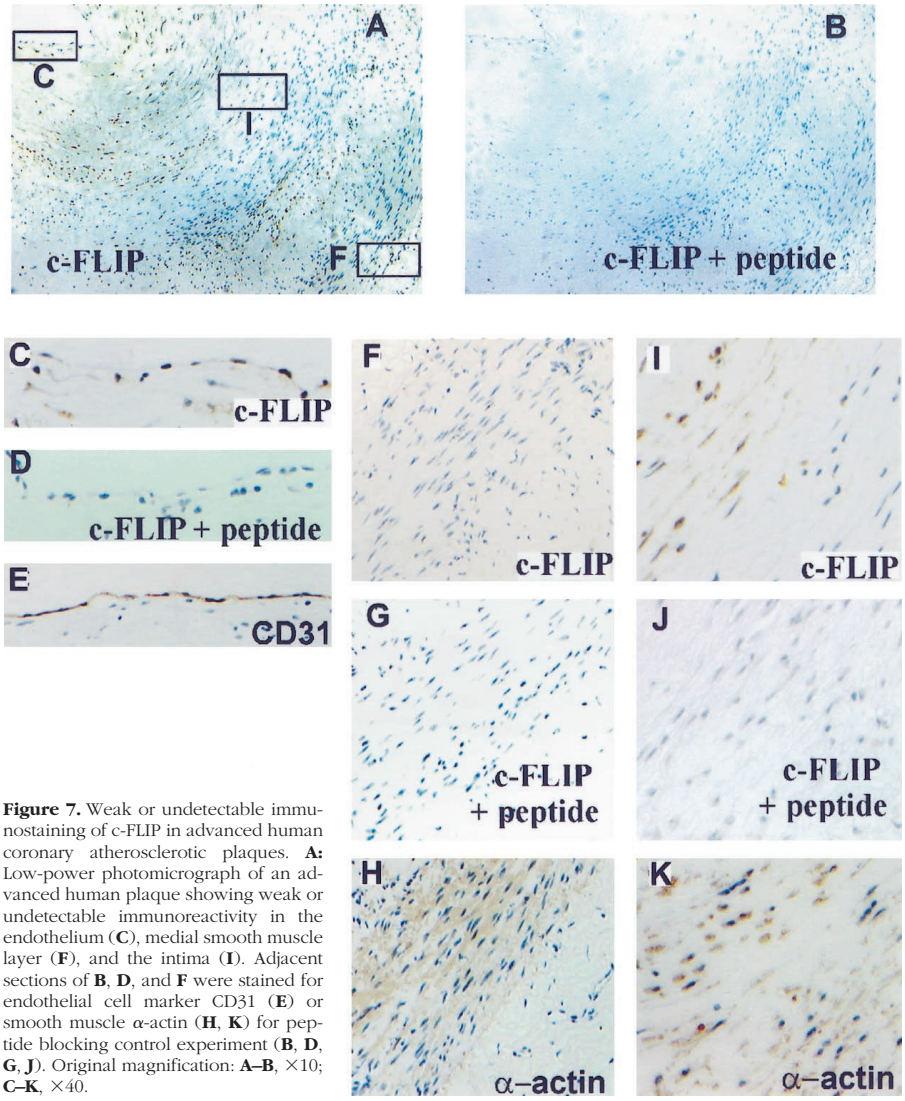

Figure 7. Weak or undetectable immunostaining of c-FLIP in advanced human coronary atherosclerotic plaques. **A:** Low-power photomicrograph of an advanced human plaque showing weak or undetectable immunoreactivity in the endothelium (**C**), medial smooth muscle layer (**F**), and the intima (**I**). Adjacent sections of **B**, **D**, and **F** were stained for endothelial cell marker CD31 (**E**) or smooth muscle α-actin (**H**, **K**) for peptide blocking control experiment (**B**, **D**, **G**, **J**). Original magnification: **A–B**, ×10; **C–K**, ×40.

correlation between TUNEL positivity and c-FLIP intensity was further carried out by counting TUNEL-positive cells in the intima, media, and endothelial lining and simultaneously grading c-FLIP intensity in the double-stained slides. As shown in Figure 9 and Table 2, the stronger c-FLIP intensity, the less TUNEL labeling index in the intima, media, and the endothelium. The majority of TUNEL-positive cells lacked c-FLIP staining, whereas cells that stain strongly for c-FLIP showed low or no TUNEL labeling, although not all of the cells that express low or undetectable levels of c-FLIP are TUNEL positive (Figure 8, A, C, and F; Figure 9). Similar results were found when we counted the number of c-FLIP-positive and -negative macrophages in TUNEL-positive or -negative zones (Table 3). These results may indicate that c-FLIP plays a role in the regulation of the death in human coronary arteries.

Discussion

Mammalian cell death is more complex than programmed cell death in the nematode, because the single caspase Ced-3 has evolved into a protease cascade that amplifies death signals initiated by multiple signaling pathways, including the death receptors and death ligands. These signaling caspases, in turn, activate executioner caspases that effect death, including Fas and FasL.[25] This paper presents data on the distribution of one regulator of these signaling caspases, c-FLIP, that suggests a role for this pathway in that of vascular wall cells. Fas has been identified in the vessel wall.[19,26] A number of *in vitro* studies show that SMC death can be induced by anti-Fas antibody or FasL in the presence of cycloheximide or additional inflammatory mediators.[27] Such death may depend on the presence or absence of apoptotic inhibitors, because systemic administration of a Fas-activating antibody in normal animals fails to induce apoptosis in the vessel wall and heart.[28] c-FLIP is a candidate for the mediator of this protective effect. Our study demonstrates that c-FLIP mRNA and protein are observed in SMCs and endothelial cells in the normal vessel wall. However, c-FLIP is down-regulated in the medial SMCs of the rat common carotid artery after angioplasty. Double staining for c-FLIP and TUNEL in the

Table 2. Clinical/Pathological Features and the Relation of Apoptosis and c-FLIP Expression in Human Coronary Arteries

Case	Age/sex	Diagnosis	Site	Histology	TUNEL-labeling index (%)			c-FLIP		
					I	M	E	I	M	E
1	57/M	ISCM	LAD	Atheroma	15.0	5.5	36.0	1+	0	0
			RCA	Atheroma	8.5	0	0	2+	2+	3+
2	53/M	ISCM	LAD	Atheroma	20.5	8.0	6.0	0	1+	0
			RCA	Atheroma	7.0	0	0	3+	2+	2+
3	63/M	ISCM	LAD	Atheroma	8.0	4.0	12.5	2+	2+	1+
			RCA	Atheroma	6.5	0	5.0	1+	2+	2+
4	57/M	ISCM	LAD	Atheroma	22.0	10.0	28.5	1+	0	0
			RCA	Atheroma	4.0	0	6.0	2+	1+	2+
5	63/M	ISCM	LAD	Atheroma	22.0	15.0	20.0	0	0	0
			RCA	Atheroma	25.0	12.5	24.0	0	1+	
6	54/M	ISCM	LAD	Atheroma	12.0	0	17.0	2+	1+	1+
			RCA	Atheroma	0	0	2.5	2+	2+	1+
7	49/M	IDCM	LAD	DIT	0.25	0	0	2+	3+	2+
			RCA	DIT	0	0	0	3+	2+	2+
8	29/F	IDCM	LAD	DIT	0	0	0	3+	3+	3+
9	58/M	IDCM	LAD	DIT	0.75	0	0	2+	3+	3+
10	61/M	IDCM	LAD	DIT	2.0	0	0	2+	3+	3+
11	37/F	IDCM	LAD	DIT	0	0	0	3+	3+	3+
12	26/F	Congenital	LAD	DIT	0	0	0	3+	3+	3+
			RCA	DIT	0	0	0	2+	3+	3+
13	34/F	Congenital	LAD	DIT	0	0	0	3+	3+	3+

Twenty-one human coronary arteries were stained by immunostaining with MAG1 antibody against c-FLIP followed by TUNEL. With the double-stained specimens, at least four sections of each arterial segment were analyzed. Ten random fields per section were examined at high magnification (×100) to determine the TUNEL-positive cells from intimal region, medial region, and endothelium (400, 400, and 200 cells per section, respectively) and the c-FLIP intensity. TUNEL-labeling index (%) was calculated by dividing number of TUNEL-positive cells by total cell number. c-FLIP intensity was semiquantitatively analzyed on a scale from 0 to 3+. ISCM, ischemic cardiomyopathy; IDCM, idiopathic dilated cardiomyopathy; Congenital, congenital heart disease; RCA, right coronary artery; LAD, left anterior descending; DIT, diffuse intimal thickening; I, vascular intima; M, vascular media; E, endothelium.

media of the rat common carotid arteries, 48 hours and 7 days after angioplasty, shows that the majority of TUNEL-positive cells lack detectable c-FLIP. The concomitant down-regulation of c-FLIP suggests that this form of death, as recently suggested for similar data for ischemia/reperfusion injury of myocardium,[16] could involve one or more of the death receptors that use caspase 8 to activate the apoptotic cascade. In contrast, our data in the intimal region of the rat carotid artery 7 days after balloon injury do not support a protective role. The quantitative counts at this time point showed that 24.3% TUNEL-positive cells were also found to be c-FLIP positive. Thus, whatever the mechanism of death in the intima at this time point, c-FLIP does not protect cells or is an inadequate protection. Medial SMCs at day 14 after the injury showed weak c-FLIP staining with a low frequency of TUNEL positivity, indicating no correlation between c-FLIP expression and TUNEL positivity.

It is interesting that c-FLIP becomes overexpressed in the neointima at later times in the rat model and is similarly and consistently prominent in nonatherosclerotic human intima. In contrast, in the human advanced atherosclerotic plaques, c-FLIP protein is very variable. In many plaques, but not all, the protein is absent or down-regulated in both endothelial cells and VSMCs. Double labeling with TUNEL and c-FLIP in human coronary arteries indicates that c-FLIP immunoreactivity in general correlates inversely with the frequency of TUNEL positivity. A few TUNEL-positive cells <4% in intima of human coronary arteries) had strong c-FLIP staining. This finding is probably because cells die by multiple pathways. c-FLIP appears to be part of a pathway that should protect against death from Fas, tumor necrosis factor receptor 1 (TNF-R1), or TNF-related apoptosis-inducing ligand (TRAIL), but not death receptor-3 (DR3).[23] Taken together, these data suggest that a c-FLIP, like Bcl-2/Ced-9-related proteins, may play a role during vascular injury and in atherosclerotic lesion formation.

It is important that evidence for the c-FLIP function in any system is at least partially inferential from its structure. Studies dependent on overexpression, on the other hand, have both protective and apoptotic effects. For example, the caspase consensus-active site cysteine of the full-length form, c-FLIP$_L$, is absent and is substituted for by a tyrosine residue. Therefore, c-FLIP is proteolytically inactive.[16] Consistent with the expected function, expression studies by a number of groups demonstrated a protective effect of c-FLIP on apoptosis induced by the death receptor ligations for TNF-R1, Fas, and TRAIL when expressed at low levels.[9,11,14,16] This inhibitory effect coincides with observations made for the viral homologs. Confusingly, other expression studies reported a marked cytotoxic effect of transfected c-FLIP by activation of caspase 8 when expressed at high levels.[10,13,15] This effect of high levels of c-FLIP could represent an artificial role of very high levels of the prodomain activity to aggregate and activate caspase 8. A possible mechanism for such an artifact appears in two studies of the overexpressed molecule. Scaffidi et al[29] found that inhibition depends on the interaction of the prodomain of c-FLIP with FADD. At normal concentrations, this interaction blocks access of caspase 8 to FADD, thus preventing formation of caspase 8 aggregates required for the activation of this enzyme. In contrast, Shu et al[10] sug-

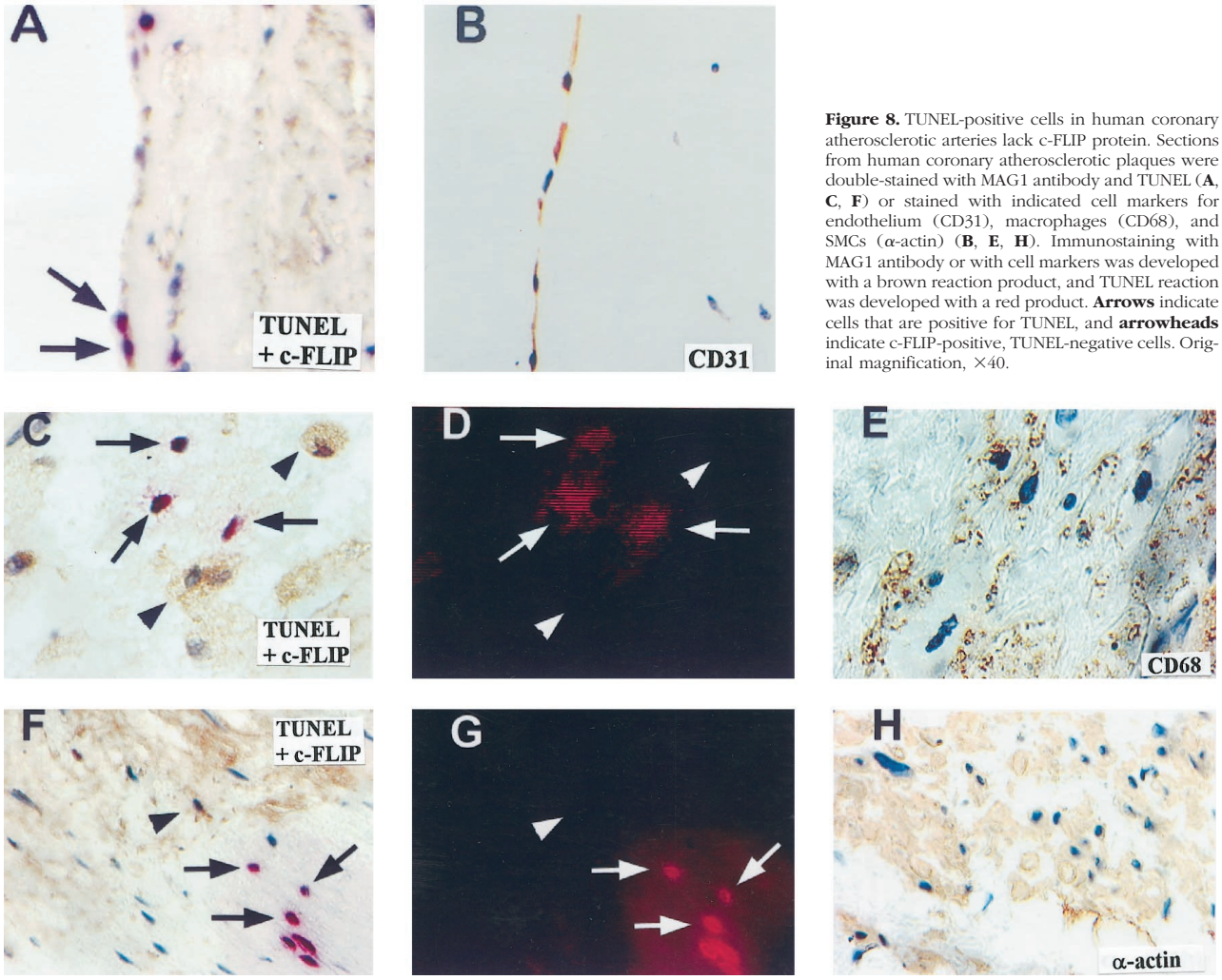

Figure 8. TUNEL-positive cells in human coronary atherosclerotic arteries lack c-FLIP protein. Sections from human coronary atherosclerotic plaques were double-stained with MAG1 antibody and TUNEL (**A**, **C**, **F**) or stained with indicated cell markers for endothelium (CD31), macrophages (CD68), and SMCs (α-actin) (**B**, **E**, **H**). Immunostaining with MAG1 antibody or with cell markers was developed with a brown reaction product, and TUNEL reaction was developed with a red product. **Arrows** indicate cells that are positive for TUNEL, and **arrowheads** indicate c-FLIP-positive, TUNEL-negative cells. Original magnification, ×40.

gested that, at high levels, c-FLIP itself might act to aggregate caspase 8. Although the role of c-FLIP in apoptotic signaling is controversial, recent biologic data support the consensus that c-FLIP is protective. v-FLIP acts as a dominant negative for FADD-mediated death, apparently by competitively binding the prodomains of caspase 8 or 10 and thus blocking Fas-mediated apoptosis.[8] Similarly, the sensitization of T cells to Fas-mediated apoptosis after T-cell antigen receptor stimulation is mediated by the down-regulation of c-FLIP, and the effect can be restored by providing cytoplasmic c-FLIP to the cells.[17] Most relevant to this paper, however, is a recent report that human umbilical vein endothelial apoptosis is dose-dependent and correlates with down-regulation of c-FLIP.[30] As with the T-cell study, this study found that the cytotoxity of oxidized lipids was reversed by transfecting endothelial cells with a c-FLIP expression plamid. These findings suggest that c-FLIP competitively inhibits binding of caspase 8 to the Fas receptor complex, thus shutting off the downstream Fas-signaling pathway.

Another candidate for a molecule protecting intimal cells from death is Bcl-XL. Perlman et al noted that induction of apoptosis in vascular media early after balloon injury coincides with down-regulation of Bcl-XL protein,

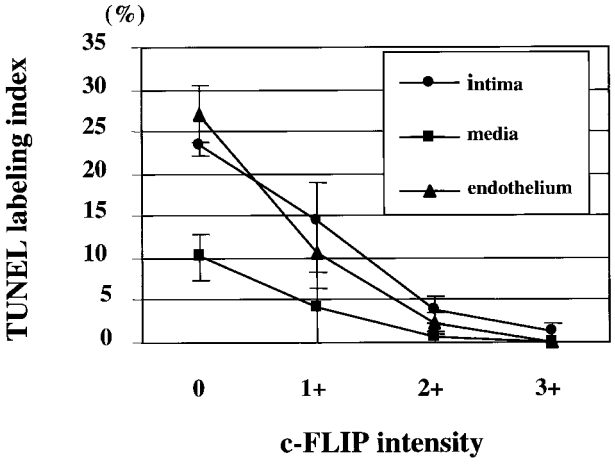

Figure 9. Inverse correlation of c-FLIP intensity and the frequency of TUNEL. The correlations between TUNEL positivity and c-FLIP intensity were examined by using 21 human coronary arteries (atheroma and DIT), the data for which were based on Table 2. Low or undetectable levels of TUNEL-labeling index are seen in regions that showed high intensity of c-FLIP (3+).

Table 3. Detailed Quantitation and Cross-Correlation of c-FLIP–Positive and –Negative Macrophages from TUNEL-Positive and -Negative Zones

	c-FLIP(+) macrophage (%)	c-FLIP(−) macrophage (%)
TUNEL(+) zones	19.4 ± 3.4	80.6 ± 3.4
TUNEL(−) zones	84.3 ± 3.1	15.7 ± 3.1

Double staining for c-FLIP and TUNEL and macrophage expression were done separately on serial sections from human atheroma. After detection in macrophage-enriched regions, we counted c-FLIP-positive and -negative cells in either TUNEL-positive zones (12 zones) or negative zones (8 zones) at the same regions, by using serial sections. TUNEL-positive zones were defined when at least five TUNEL-positive cells exist at high magnification (×100). Approximately 200 cells were counted per zone. Results are expressed in percentages of c-FLIP-positive and -negative cells and corresponding standard errors.

an antiapoptotic molecule, assessed by immunohistochemistry in the arterial media.[31] Pollman et al showed that the neointima after balloon injury stained uniformly to Bcl-XL, although some cell death could be observed.[32] They also showed that an antisense Bcl-XL construct is able to promote thinning of the neointima after balloon injury. We have shown previously that c-FLIP interacts with Bcl-XL,[13] suggesting that these antiapoptotic molecules may interact in controlling cell death in the intima.

It is important that these *in vivo* studies of antiapoptotic molecules, except for the antisense studies of Bcl-XL, are phenomenological. The absence of c-FLIP in dying cells, indicated by simultaneous staining with TUNEL and an antibody, may be a consequence of protease generated during death rather than a cause. Although we have no direct way of answering this concern, *in vitro* studies with death receptor-activated death in lymphocytes show that c-FLIP is truncated to a form still recognized by the MAG-1 antibody or antibodies directed at the same peptide sequence.[17] A further caution is the use of TUNEL to identify dead cells. TUNEL data clearly depend on the method used. For example, the reported value of TUNEL-labeled cells obtained from atherosclerotic plaques ranged from less than 2% up to 60%.[33] In addition, Geng et al suggested that, if the apoptotic bodies are not removed or remain TUNEL positive after engulfment, then high labeling indices may be independent of the time course of cell death itself.[2] Thus, TUNEL positivity probably only reflects occurrence of death rather than giving an absolute value.[34] To provide experiment-to-experiment consistency in the current study, however, TUNEL analyses were always performed including human tonsil and rat thymus as positive controls of human and rat samples, respectively.

In summary, c-FLIP is widely expressed in the normal vessel wall and down-regulated in the media of the rat common carotid artery after balloon injury and in human atherosclerotic atheroma. The selective expression of c-FLIP in rat neointimal lesions and in diffuse intimal thickening is consistent with previous studies that have demonstrated that intimal cells exhibit a differential pattern of gene expression.[35] For human atherosclerotic plaques, the majority of TUNEL-positive cells lack detectable c-FLIP. Loss of c-FLIP in atherosclerotic plaques may be an important step in the ultimate breakdown of the atherosclerotic lesion.

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