Advanced Glycation End Products Enhance Expression of Pro-apoptotic Genes and Stimulate Fibroblast Apoptosis through Cytoplasmic and Mitochondrial Pathways*

Received for publication, June 7, 2004, and in revised form, November 29, 2004
Published, JBC Papers in Press, December 6, 2004, DOI 10.1074/jbc.M406313200

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Both aging and diabetes are characterized by the formation of advanced glycation end products (AGEs). Both exhibit other similarities including deficits in wound healing that are associated with higher rates of fibroblast apoptosis. In order to investigate a potential mechanism for enhanced fibroblast apoptosis in diabetics and aged individuals, experiments were carried out to determine whether the predominant advanced glycation end product in skin, Nε-(carboxymethyl) lysine (CML)-collagen, could induce fibroblast apoptosis. In vivo experiments established that CML-collagen but not unmodified collagen induced fibroblast apoptosis and that apoptosis was dependent upon caspase-3, -8, and -9 activity. In vitro experiments demonstrated that CML-collagen but not control collagen induced a time- and dose-dependent increase in fibroblast apoptosis. By use of blocking antibodies, apoptosis was shown to be mediated through receptor for AGE signaling. AGE-induced apoptosis was largely dependent on the effector caspase, caspase-3, which was activated through both cytoplasmic (caspase-8-dependent) and mitochondrial (caspase-9) pathways. CML-collagen had a global effect of enhancing mRNA levels of pro-apoptotic genes that included several classes of molecules including ligands, receptors, adaptor molecules, mitochondrial proteins, and others. However, the pattern of expression was not identical to the pattern of apoptotic genes induced by tumor necrosis factor α.

Advanced glycation end products (AGEs) result from non-enzymatic reactions of carbohydrates and oxidized lipids with proteins (1, 2). Lysine side chains condense with aldehyde or ketone structures to form a reversible Schiff's base. This structure isomerizes to a more reactive ketoamine (Amadori product), which then may undergo additional rearrangement, dehydration, condensation, and oxidation reactions. These structures can result in protein cross-links and in a variety of protein modifications that collectively are known as advanced glycation end products (3). Nε-(Carboxymethyl) lysine (CML) structure is a prevalent AGE and is generated principally by oxidative cleavage of Amadori intermediates (3).

AGEs form in tissues and accumulate during aging. AGE formation occurs throughout life, and AGEs are found at significantly higher levels in aged individuals (4, 5). Their formation is considered to be one of the major etiologic factors in the pathophysiology of aging (6–8). AGEs have also been studied in relation to development of many age-related chronic diseases including cataract formation, neurodegenerative disorders such as Alzheimer disease, osteoarthritis and changes observed in myocardial dysfunction (9–14).

AGE formation is enhanced by hyperglycemia and has been linked to many of the complications of diabetes. By functionally blocking AGE activity or by applying AGEs in vivo, it has been shown that AGEs contribute to several pathologic processes. These include diabetes-associated cataract formation, nephropathy, retinopathy, neuropathy, and periodontal disease (15–20). AGEs also impair osseous wound healing (21).

Originally it was thought that most of the effects of AGEs were the result of cross-linking of matrix and other proteins (3). Although cross-linking is physiologically important, glycation can alter proteins so that they acquire signaling properties, which they do not otherwise possess. Several AGE cell surface receptors/binding proteins have been identified. The most thoroughly studied is receptor for advanced glycation end products (RAGE). RAGE is a multi-ligand member of the immunoglobulin superfamily of cell surface receptors that binds many ligands including those of the S100/calgranulin family (22). Other receptors/binding proteins include macrophage scavenger receptor types I and II, oligosaccharidyl transferase-48 (AGE-R1), 80K-H phosphoprotein (AGE-R2), and galectin-3 (AGE-R3). These are expressed on a wide range of cells including smooth muscle cells, monocytes, macrophages, endothelial cells, podocytes, astrocytes, microglia, and fibroblasts. Most of the biologic activities associated with AGEs have been shown to be transduced by RAGE, whereas the scavenger receptors are thought to regulate removal of AGEs (23, 24). AGEs can modulate inflammatory events by stimulating production of reactive oxygen species, chemotaxis, activation of monocytes/macrophages, and stimulation of interleukin-1 and TNF production (25–30).

It is well known that AGEs form in the skin as part of the aging process and as a result of diabetes (31–33). Accumulation...
of AGES in skin has been related to tissue stiffening and lack of elasticity during aging (5, 31, 32). However, AGES in connective tissue can have detrimental effects through cell signaling. For example, RAGE activation in dermal fibroblasts reduces collagen synthesis and matrix production (34). AGES also contribute to impaired diabetic wound healing, in part through interfering with formation of an extracellular matrix (35).

One mechanism through which AGES may affect pathologic processes is by enhanced apoptosis as supported by in vitro studies. AGES are pro-apoptotic for cultured retinal pericytes, corneal endothelial cells, neuronal cells, and renal mesangial cells (36–38). The mechanisms by which AGES lead to apoptosis are not well understood. There are reports suggesting that AGES may enhance apoptosis indirectly through increasing oxidative stress or via induced expression of pro-apoptotic cytokines (36, 38, 39). Interestingly, enhanced apoptosis of these cells is also associated with diabetic complications such as retinopathy, neuropathy, nephropathy, and accelerated vasculopathy (38–40). Enhanced apoptosis has been linked to many of the detrimental effects of aging, which is also associated with AGE accumulation (41). Because fibroblasts play important roles in the maintenance and healing of dermal connective tissue, the accumulation of AGES in skin may have a detrimental effect, in part, through promoting fibroblast apoptosis. To address this issue, we investigated the apoptotic effect of AGES on fibroblasts and the mechanisms through which AGES induce apoptosis in these cells.

**MATERIALS AND METHODS**

**CML-collagen**—CML-collagen was prepared by chemical modification of acid-soluble bovine skin collagen (Sigma), as described previously (21, 42). Briefly, 50 mg of collagen was dissolved in 25 ml of 1 mM HCl freshly made in sterile water and incubated at 37 °C with occasional mixing. Sterile PBS (25 ml) was added, followed by sodium cyanoborohydride (1.42 g) and glyoxylic acid (0.715 g). Control collagen was prepared at the same time, except that no glyoxylic acid was added. All samples were then incubated at 37 °C for 24 h. AGE collagen and control collagen were then exhaustively dialyzed against distilled water. In dose-response experiments where higher concentrations of CML-collagen were used, AGE and control collagen was dialyzed against PBS. Both CML-collagen and control collagen were soluble at the concentrations stored and tested. In total, 3–8% of lysine residues in CML-collagen were converted to CML, as determined by the trinitrobenzenesulfonyl acid assay (43). The percentage of modification of collagen that we have generated is 10-fold less than the amount used in a recent report to assess CML binding and activation of NF-kB (42) and only a small amount higher than that reported for the skin of aged or diabetic individuals (31). CML-collagen was highly reactive on Western blots with anti-CML monoclonal antibody 6D12 (Wako, Richmond, VA), whereas control collagen was not reactive. The amount of endotoxin contamination was measured by Pyrochrome Limulus Amebocyte Lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA) and found to be low (0.094 ± 0.01 ng/mg lipopolysaccharide in control collagen and 0.087 ± 0.01 ng/mg lipopolysaccharide in CML-collagen).

**Animals**—CD1 mice were purchased from Charles River Laboratories, (Waltham, MA). These are outbred mice that were selected because they do not exhibit strain-associated responses, as has been reported in some cases where a particular strain of mouse has been studied (45, 46). All procedures involving mice were approved by the Boston University Medical Center Institutional Animal Care and Use Committee. Mice were anesthetized with injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) in sterile PBS. CML-collagen or unmodified collagen was injected into the loose connective tissue adjacent to calvarial bone at a point on the midline of the skull located between the ears. Injection at this anatomic site can be reproducibly achieved. For each data point, there were six mice (n = 6). We undertook preliminary experiments to identify a dose for CML-collagen that gave a moderate number of apoptotic cells. On this basis, 100 μg of CML-collagen or an equal amount of unmodified collagen was injected. Mice were euthanized 24 h after injection. In addition to CML-collagen or unmodified collagen alone, some animals were treated by intraperitoneal injection of caspase-3, -8, or -9 inhibitor (1 mg/kg) 1 h before CML-collagen injection and locally (25 μg) at the time of CML-collagen injection. The caspase-3 inhibitor Z-DEVD-fmk, the caspase-8 inhibitor Z-IETD-fmk, and the caspase-9 inhibitor Z-LEHD-fmk were purchased from R&D Systems (Minneapolis, MN). Control mice received CML-collagen or control collagen at the same time, except that no glyoxylic acid was added.

**FIG. 1. AGE stimulates fibroblast apoptosis in vivo.** A, histologic sections at the site of injection in the mouse scalp. Top panel, TUNEL staining of specimens 24 h after injection with unmodified collagen; bottom panel, TUNEL staining 24 h after injection of CML-collagen. Large arrow points to apoptotic cell, whereas small arrows point toward normal cells not stained with nuclear fast red. B, quantiative analysis of fibroblast apoptosis after CML-collagen injection. The number of fibroblastic TUNEL-positive apoptotic cells was counted in specimens 24 h after injection of CML-collagen, unmodified collagen, or the control, zero time point. Each value represents the mean of six specimens ± S.E. The experiment was performed twice with similar results.
unmodified collagen containing 2% Me2SO (Sigma-Aldrich).

Preparation of Histologic Sections—Animals were euthanized by decapitation, and their heads were fixed for 72 h in cold 4% paraformaldehyde and decalciﬁed by incubation with cold Immunocal (Decal Corp., Congers, NY) for ~12 days with solution changed daily. Parafﬁn-embedded sagittal sections were prepared at a thickness of 5–6 μm.

TUNEL Assay and Quantitative Histologic Analysis—Apoptotic cells were detected by an in situ TUNEL assay by means of a TACS 2 TdT-Blue Label kit purchased from Trevigen (Gaithersburg, MD), following the manufacturer’s instructions. Sections were counterstained with nuclear fast red. The number of ﬁbroblastic apoptotic cells was determined in six specimens per group by counting the number of TUNEL-positive cells that had the characteristic microscopic appearance of ﬁbroblasts. Counts and measurements were conﬁrmed by reanalysis of all the specimens by an independent examiner. The intra- and inter-examiner variations were <10%. Student’s t-test was used to determine signiﬁcant differences between the experimental and control groups at the p < 0.05 level.

Caspase Activity—Caspase-3, -8, and -9 activities from in vitro and in vivo experiments were assayed with fluorometric kits purchased from R&D Systems. Brieﬂy, after sacrifice at the indicated time points, murine scalps were immediately dissected from the calvaria and frozen in liquid nitrogen. Frozen tissues were pulverized, and lysates were prepared using cell lysis buffer provided by R&D Systems. After centrifugation, total protein was quantitated using a BCA protein assay kit (Pierce). Caspase-3 activity was detected by using the speciﬁc caspase-3 ﬂuorogenic substrate, DEVD peptide conjugated to 7-amino-4-triﬂuoromethyl coumarin (AFC). Caspase-8 activity was detected by using the speciﬁc caspase-8 ﬂuorogenic substrate, IETD-AFC. Caspase-9 activity was detected by using the speciﬁc caspase-9 ﬂuorogenic substrate, LEHD-AFC. Measurements were made on a ﬂuorescent microplate reader using ﬁlters for excitation (400 nm) and detection of emitted light (505 nm). In some assays, recombinant caspase-3 enzyme (R&D Systems) was used as a positive control. Buffers without cell lysate and cell lysate without substrate were used as negative controls. The same caspase-3, -8, and -9 inhibitors were used in both in vitro and in vivo studies. In addition, the pan-caspase (general caspase) inhibitor Z-VAD-fmk was purchased from R&D Systems for in vitro assays. For each group, there were six specimens (n = 6).

Cell Culture—Primary human adult dermal ﬁbroblasts were purchased from Cambrex (Walkersville, MD). Cells were propagated and maintained in Dulbecco’s modiﬁed Eagle’s medium (Cambrex) supplemented with 0.5% fetal bovine serum. Assays were performed when the cultures reached 75–85% conﬂuence. In most experiments, 200 μg/ml CML-collagen or unmodiﬁed collagen was used, which is equivalent to 2 μM. Apoptosis of ﬁbroblasts was determined by an ELISA technique measuring histone-associated DNA fragments (Roche Applied Science), following the manufacturer’s instructions. In these studies, 20,000 ﬁbroblasts/cm2 were treated with CML-collagen (200 μg/ml) or unmodiﬁed collagen (200 μg/ml) for 24 h. Apoptosis was determined by ELISA, and the cell numbers were assessed in corresponding wells to normalize apoptosis measurements.

For caspase activity measurements, the same technique was used as described above for in vitro studies. In some cases, cells were treated with caspase-3, caspase-8, caspase-9, caspase-8 + caspase-9, or pan-caspase inhibitors (50 μM) at the time of CML-collagen stimulation. The dose of each inhibitor was selected based on our previous results (47).
Control cells were incubated in assay medium supplemented with vehicle alone (2% Me2SO) in the culture media.

Inhibition of RAGE—Antibodies to the extracellular domain of RAGE have been shown to inhibit binding of CML modified proteins to RAGE and have been used to assess the impact of RAGE activation of cellular events (42, 48). To study the role of RAGE, fibroblast cultures were incubated with CML-collagen or unmodified collagen (200 μg/ml) in the presence or absence of anti-RAGE polyclonal antibodies specific for the extracellular domain of RAGE (10 μg/μl) or non-immune serum (10 μg/μl) for 24 h (Biocompare, San Francisco, CA). As controls, cells were incubated with 10 μg/ml RAGE antisera or non-immune serum without AGE stimulation. The extent of apoptosis was determined by ELISA. Statistical difference between samples was determined by one-way analysis of variance followed by Tukey’s multiple comparison tests.

EMSA—Fibroblast cultures were incubated in assay medium for 1 h with 200 μg/ml CML-collagen or unmodified collagen in the presence or absence of a specific NF-κB inhibitor, SN50 (100 μg/ml) (Biomol, Plymouth Meeting, PA). Nuclear proteins were extracted using protein extraction kit (Pierce) following the manufacturer’s instructions. Concentrations of nuclear proteins were measured by using BCA protein assay kit (Pierce). Interaction between NF-κB in the protein extract and DNA probe was investigated using EMSA kit from Panomics (Redwood City, CA) following the manufacturer’s instruction.

Microarray—Fibroblast cell cultures were exposed to CML-collagen (200 μg/ml) or TNF-α (20 ng/ml) for 6 h. TNF-α was purchased from R&D Systems. Control cells were exposed to either unmodified collagen (200 μg/ml) or vehicle alone (PBS). RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). Human Apoptosis GEArray Q series kit that includes 96 key genes involved in apoptosis was purchased from SuperArray, Inc. (Bethesda, MD). Total RNA was used as the template for 32P-labeled cDNA probe synthesis following the manufacturer’s instructions. After overnight hybridization with labeled probe, the GEArray membranes were exposed using a PhosphorImager. The resulting digital image was converted to a raw data file using Scanalyze software (www.microarray.org/software.html). GEArray Analyzer software (SuperArray, Inc.) was used for data analysis. The microarray was performed twice with similar results, and the mean values of the two experiments are presented for each gene. The microarray for TNF-α stimulation was performed simultaneously with the AGE stimulation array.

RT-PCR—Five μg of total RNA was used to produce cDNA using a First Strand cDNA Synthesis Kit (SuperArray, Inc.). PCR was performed on the synthesized cDNA using the SingleGene PCR Kit purchased from SuperArray, Inc. following the manufacturer’s protocol. Specific primers were used for each gene, and GAPDH was used as internal control. This kit has the advantage that GAPDH and the gene of interest are amplified in the same tube and for the same number of cycles. Based on a pilot study, 24 and 28 cycles were chosen to compare the gene expression. After electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide, bands were visualized on a UV-box. The density of each band was measured and compared using Quantity One software (Bio-Rad). The optical density of each band was normalized by the value of the GAPDH in the same lane. Two separate RT-PCRs were performed with similar results. Student’s t test was used to determine significant differences between the experimental and control groups.

RESULTS

In Vivo Induction of Apoptosis by CML-collagen—To study the effect of AGEs on fibroblast apoptosis in vivo, 100 μg of CML-collagen or control collagen was injected in the scalp of CD1 mice. Histologic sections were examined for fibroblast apoptosis by their characteristic appearance in the TUNEL assay (Fig. 1). Fibroblast apoptosis could be detected in the CML-collagen-treated group. In comparison, there was virtually no induction of fibroblast apoptosis in mice treated with unmodified collagen compared with the zero time point (Fig. 1A). Quantitative analysis indicated that CML-collagen stimulated a 4-fold increase in apoptotic cells compared with un-
modified collagen (Fig. 1B) \( p < 0.05 \). When expressed as the percentage of TUNEL-positive fibroblasts, the levels were \( 0.85 \pm 0.05 \) in the CML-collagen-inoculated group and \( 0.21 \pm 0.01 \) \( p < 0.05 \) for control collagen-treated group.

In Vitro Induction of Apoptosis by CML-collagen—In vitro studies were carried out to measure the direct effect of AGEs on fibroblast apoptosis under well-defined conditions. Apoptosis of human adult primary skin fibroblasts was measured by the relative amount of histone-associated DNA fragments in the cytoplasm as determined by ELISA. A time course experiment determined that AGE-induced apoptosis was not detected before 6 h (Fig. 2A). At the 6 h time point, a 1.7-fold increase was noted, whereas at 24 h, a 3-fold increase in apoptosis was detected. The increase at 6 and 24 h was statistically significant (\( p < 0.05 \)). CML-collagen induced a dose-dependent increase in fibroblast apoptosis, whereas control collagen did not (Fig. 2B). At 200 \( \mu \)g/ml, CML-collagen compared with unmodified collagen induced a 3-fold increase in apoptosis, which was statistically significant (\( p < 0.05 \)). Saturating levels were reached at 400 \( \mu \)g/ml CML-collagen with a 5-fold increase in apoptosis (\( p < 0.05 \)). In subsequent studies, a concentration of 200 \( \mu \)g/ml CML-collagen or control collagen and a time point of 24 h were used unless stated otherwise.

Role of RAGE in Induction of Apoptosis by CML-collagen—To determine the significance of RAGE activation in the apoptotic response to AGEs, human adult dermal fibroblasts were incubated with CML-collagen (200 \( \mu \)g/ml) in the presence or absence of anti-RAGE polyclonal antibodies specific for the extracellular domain of RAGE (10 \( \mu \)g/ml) or non-immune serum (10 \( \mu \)g/ml) for 24 h (Fig. 3). CML-collagen stimulated a significant increase in fibroblast apoptosis (\( p < 0.05 \)). However, anti-RAGE antibody was able to completely block the apoptotic effects of AGE so that there was no difference between fibroblasts incubated with control collagen and cells incubated with CML-collagen plus RAGE antibody. Matched pre-immune serum had no effect on CML-collagen-induced apoptosis, demonstrating specificity of the antibody. As additional controls, cells incubated with RAGE antibody or pre-immune serum in the absence of CML-collagen had no direct effect on apoptosis. The results obtained are consistent with functional studies in which RAGE antibody inhibited activation of cells by CML-albumin (data not shown).

In Vitro Effects of CML-collagen on Activation of Caspases-3, -8, and -9—To further investigate mechanisms of AGE-induced fibroblast apoptosis, the activation of initiator and executioner caspases was measured in adult human fibroblasts after stimulation by CML-collagen or unmodified collagen (Fig. 4). CML-collagen stimulated a 5-fold increase in caspase-3, a 4.3-fold increase in caspase-8, and a 3.2-fold increase in caspase-9 activity compared with cells treated with unmodified collagen alone. Each was statistically significant (\( p < 0.05 \)).

Effects of Different Caspase Inhibitors on Apoptosis in Vitro—To investigate the effect of caspase-3, caspase-8, and caspase-9 activity on AGE-induced apoptosis in human dermal fibroblasts, cells were incubated with a pan-caspase and caspase-3, -8, or -9 inhibitors at the time of CML-collagen stimulation (Fig. 5A). The concentration of inhibitors used was 50 \( \mu \)M, based on previous studies that demonstrated that this concentration inhibits virtually all activity in a highly specific manner (49–52). The pan-caspase inhibitor reduced apoptosis compared with CML-collagen alone by 92%. Caspase-3 inhibitor reduced the apoptotic effect of CML-collagen by 85%, a level similar to that of the pan-caspase inhibitor. Caspase-8 inhibitor alone and caspase-9 inhibitor alone significantly reduced apoptosis by 65% and 32%, respectively (\( p < 0.05 \)). However, when caspase-8 and -9 inhibitors were combined, the result was similar to that of the caspase-3 inhibitor (\( p > 0.05 \)). Taken together, these results suggest that AGEs function primarily through caspase-3, whose induction can be accounted for by activation from both caspases-8 and-9. The latter is demonstrated by (\( p < 0.05 \)), establishing that caspase-3 activity is significantly reduced in the presence of caspase-8 and -9 inhibitors (\( p < 0.05 \)) (Fig. 5B).

Role of NF-\( \kappa \)B in AGE-stimulated Apoptosis—To investigate the effect of AGE on activation of NF-\( \kappa \)B in fibroblasts, adult human dermal fibroblasts were incubated with CML-collagen or unmodified collagen for 1 h. Using EMSA, it was shown that CML-collagen but not unmodified collagen induced NF-\( \kappa \)B activation (Fig. 6A). This effect could be blocked completely by an NF-\( \kappa \)B inhibitor SN50 (100 \( \mu \)g/ml). To investigate the role of NF-\( \kappa \)B activation in AGE-induced apoptosis, adult human dermal fibroblasts were treated with CML-collagen and SN50 simultaneously, and apoptosis was measured by ELISA. Inhibition of NF-\( \kappa \)B did not suppress fibroblast apoptosis (Fig. 6B). In fact, the level of apoptosis was higher in the presence of SN50 inhibitor (\( p < 0.05 \)).

In Vitro Effect of CML-collagen on Expression of Apoptotic Genes and Comparison with TNF—Experiments with microarrays were carried out to study the impact of AGE on expression of apoptosis-related genes. For the purpose of this analysis, a minimum 2-fold change in the level of expression was used as a threshold (Tables I and II). In addition, microarrays were also carried out under identical conditions to compare AGE modulation of apoptotic genes with that of TNF-\( \alpha \). As shown in Table
Comparison of apoptotic gene expression in fibroblasts in vitro stimulated by CML-collagen or TNF-α. Primary human dermal fibroblasts were incubated with 200 μg/ml CML-collagen or 20 μg/ml TNF-α. Cells exposed to unmodified collagen or PBS were used as controls. Total RNA was isolated 6 h after stimulation and subjected to microarray analysis. Left panels show genes whose expression is up-regulated >2-fold by both AGE and TNF stimulation. Right panels show genes that were differentially regulated by AGE or TNF, i.e. AGE or TNF but not both induced a 2-fold change in mRNA level.

| Gene          | AGE     | TNF     | AGE/TNF ratio |
|---------------|---------|---------|---------------|
| LTB          | 5.01    | 6.41    | 0.78          |
| TNF          | 3.74    | 5.84    | 0.64          |
| OX40L        | 3.56    | 2.51    | 1.42          |
| TNFSF9       | 3.03    | 5.18    | 0.58          |
| APRIL        | 2.85    | 3.38    | 0.84          |
| CD30L        | 2.27    | 2.01    | 1.15          |
| TNF          | 3.74    | 5.84    | 0.64          |
| LTB          | 5.01    | 6.41    | 0.78          |
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| OX40L        | 3.56    | 2.51    | 1.42          |
| TNFSF9       | 3.03    | 5.18    | 0.58          |
| APRIL        | 2.85    | 3.38    | 0.84          |
| CD30L        | 2.27    | 2.01    | 1.15          |
| TNFRSF9      | 7.67    | 3.12    | 2.46          |
| Fas          | 4.49    | 6.19    | 0.73          |
| CD27         | 2.61    | 3.87    | 0.67          |
| LTgR         | 2.61    | 2.80    | 0.93          |
| CD40         | 2.11    | 2.23    | 0.95          |
| TNFRSF9      | 7.67    | 3.12    | 2.46          |
| Fas          | 4.49    | 6.19    | 0.73          |
| CD27         | 2.61    | 3.87    | 0.67          |
| LTgR         | 2.61    | 2.80    | 0.93          |
| CD40         | 2.11    | 2.23    | 0.95          |
| Mitochondrial|         |         |               |
| Bcl-2        | 5.84    | 4.72    | 1.24          |
| MCL-1        | 4.36    | 4.29    | 1.02          |
| Bax          | 2.15    | 2.45    | 0.88          |
| Nip-3        | 2.11    | 3.40    | 0.62          |
| Caspases     |         |         |               |
| Caspase-9    | 3.13    | 2.92    | 1.07          |
| Caspase-8    | 3.07    | 3.50    | 0.88          |
| Caspase-6    | 2.68    | 3.02    | 0.89          |
| Caspase-3    | 2.52    | 3.12    | 0.81          |
| Caspase-1    | 2.48    | 2.51    | 0.99          |
| Caspase-2    | 2.19    | 3.52    | 0.62          |
| Caspase-7    | 2.16    | 3.21    | 0.67          |
| Caspase-4    | 2.05    | 2.16    | 0.95          |
| Adaptors     |         |         |               |
| TRAF2        | 6.52    | 3.52    | 1.85          |
| CASPER       | 5.64    | 7.16    | 0.79          |
| TRAF1        | 4.45    | 8.34    | 0.53          |
| RIP          | 2.57    | 3.35    | 0.77          |
| I-TRAF       | 2.50    | 3.74    | 0.67          |
| FADD         | 2.23    | 3.27    | 0.68          |
| Others       |         |         |               |
| GADD45       | 8.00    | 7.81    | 1.02          |
| P53          | 6.58    | 5.42    | 1.21          |
| Cardiac/Rip2 | 3.65    | 4.53    | 0.81          |
| Chk2/RAD53   | 3.52    | 3.50    | 1.01          |
| Mdm2         | 2.52    | 2.47    | 1.02          |
| IAP-2        | 2.30    | 2.35    | 0.98          |
| BCL-10       | 2.25    | 3.06    | 0.74          |
| Adaptors     |         |         |               |
| TRAF3/CRAF1  | 0.31    | 1.27    | 0.24          |
| TRAF4        | 0.09    | 2.39    | 0.04          |
| TRAF5        | 0.45    | 1.54    | 0.29          |
| Bcl-x        | 1.38    | 0.25    | 5.52          |
| Others       |         |         |               |
| Nop30        | 0.45    | 0.74    | 0.61          |
| ATM          | 0.38    | 1.10    | 0.35          |

I. CML-collagen compared with unmodified collagen induced a 2–5-fold increase in the expression of six members of the TNF ligand superfamily (LT-β, TNF-α, OX40L, TNFSF9, APRIL, and CD30L). The same six ligands had enhanced mRNA levels stimulated by TNF. Another six TNF superfamily ligands (CD27L, CD40L, Lta, TRAIL, TNFSF12, and FasL) were induced more than 2-fold by TNF-α stimulation, but not by AGE. From the TNF receptor family of the genes, Fas, CD27, LTβR, CD40, and TNFSF9 mRNA levels increased 2–7-fold in response to both AGE and TNF-α. However, other members of this family exhibited increased expression by TNF-α but not AGE stimulation (TRAIL-R3, DR3, TNFRSF14, TNFR1, TNFR2, and OX40). Of the mitochondrial proteins that regulate apoptosis, AGES and TNF both increased Bax, Bcl-2, and Nip-3, which are pro-apoptotic members of the Bcl-2 family, and MCL-1, which is an anti-apoptotic member of the Bcl-2 family. In addition, TNF-α enhanced the pro-apoptotic proteins Blk, Bak, and Bad, whereas AGE had no significant effect on expression of these genes. The caspase family showed significant increases in expression after both stimuli. Initiator caspases-2, -8, and -9 and effector caspases -3, -6, and -7 showed a 2–3-fold increase by both AGE and TNF. From the adaptor family, TRAF2, CASPER, TRAF1, RIP, I-TRAF, and FADD increased between 2- and 8-fold in response to both stimuli. The genes of six adaptor molecules (Flash, Apaf-1, DAP-kinase, myD88, CRADD, and TRAF6) increased more than 2-fold in response to TNF but not to AGE. P53, a cell cycle...
To confirm the results of the microarray, RT-PCR using specific primers for five selected genes of interest was examined (Fig. 7). Amplification was performed over 24 and 28 cycles with similar results.

![Table III](image)

| TNF ligand family | TNF receptor family | Bel-2 family | Caspases | Adaptor | Other |
|-------------------|---------------------|--------------|----------|---------|-------|
| TNFSF14           | TRAIL-R, Trail receptor (DR5), TRAIL-R4, and CD90 | Biml, HRK, Bel-w, and Bik | Caspase-10, caspase-5, caspase-14, and caspase-13 | Bar and Trip | CIDE-B, DFF40, DFFA, Chk1, IAP-1, Nod/CARD4, Apollon/Bruce, survivin, and NAIP/BIRC1 |

![Table IV](image)

| Gene          | CML-collagen/control | TNF/control |
|---------------|-----------------------|-------------|
|               | 24 cycles 28 cycles   | 24 cycles 28 cycles |
| Bcl-2         | 0.43 0.49            | 0.42 0.44   |
| Bax           | 2.23 2.02            | 2.24 2.13   |
| TRAIL         | 1.5 1.01             | 2.58 2.74   |
| P53           | 3.43 3.72            | 3.15 3.33   |
| TNF SF 9     | 2.8 3.06             | 2.8 2.7     |

In Table II, molecules down-regulated more than 2-fold by AGE and/or TNF stimulation are shown. Expression of the anti-apoptotic gene Bcl-2 was diminished twice with similar results. In order to identify the mechanisms through which AGE-induced apoptosis was signaled, we identified the receptor in vitro data.

DISCUSSION

To study the apoptotic effect of AGE stimulation on fibroblasts and also to investigate mechanisms through which AGEs induce apoptosis in these cells, CML-collagen was injected subcutaneously in the scalps of mice. The direct effects of AGE stimulation on fibroblasts were examined in culture. The results establish that AGEs significantly induce apoptosis in fibroblasts in vivo and in vitro and that it is largely dependent upon caspase-8 and -9 activation of caspase-3. To our knowledge, these studies are the first to characterize the caspase pathways through which AGEs induce apoptosis in vivo and in vitro. Moreover, AGE stimulation has a predominant effect of enhancing expression of pro-apoptotic genes.

In order to identify the mechanisms through which AGE-induced apoptosis was signaled, we identified the receptor invertebrate. The pro-apoptotic, adaptor molecule TRAF4 mRNA levels decreased almost 10-fold in response to AGE while increasing 2-fold after TNF stimulation. Adaptor molecules TRAF3 and TRAF5 decreased in response to AGE but did not change more than 2-fold after TNF stimulation. The mRNA level of the anti-apoptotic gene Bcl-x was diminished 4-fold by TNF but not by AGE. Two other pro-apoptotic genes, Nop30 and ATM, were down-regulated by AGE but not by TNF. A list of other apoptotic genes whose expression was not enhanced or diminished more than 2-fold is shown in Table III.

To confirm the results of the microarray, RT-PCR using specific primers for five selected genes of interest was examined (Fig. 7). Amplification was performed over 24 and 28 cycles to establish that differences obtained were not an artifact of a particular number of amplifications. Densitometric analysis of the resulting bands indicates that the RT-PCR results confirmed the data obtained for the microarray very well (Table IV). In every case, the RT-PCR results exhibited the same 2-fold change or lack of 2-fold change in cells stimulated by CML-collagen or TNF-α compared with the respective control obtained in two separate experiments.

In Vivo Effects of CML-collagen on Activation of Different Caspases—To determine whether AGEs also induce caspase activity in vivo, experiments were performed in which CML-collagen and unmodified collagen were injected into the scalp, and the activation of caspases was measured (Fig. 8A). CML-collagen (100 μg) increased caspase-3 activity 2.5-fold, increased caspase-8 activity 2.4-fold, and increased caspase-9 activity 2.2-fold compared with unmodified collagen (p < 0.05).

To study the functional role of caspase-8 and -9 in AGE-induced apoptosis, mice received injection with CML-collagen with or without specific caspase inhibitors (Fig. 8B). Caspase-8 inhibitor decreased AGE-induced fibroblast apoptosis in vivo 77%, whereas caspase-9 inhibitor decreased it by 43% (p < 0.05). These results agree well with in vitro data.

regulator that is strongly pro-apoptotic, increased almost 6-fold in both groups.

In Table II, molecules down-regulated more than 2-fold by AGE and/or TNF stimulation are shown. Expression of the anti-apoptotic gene Bcl-2 was reduced more than 2-fold by both AGE and TNF. The pro-apoptotic, adaptor molecule TRAF4 mRNA levels decreased almost 10-fold in response to AGE while increasing 2-fold after TNF stimulation. Adaptor molecules TRAF3 and TRAF5 decreased in response to AGE but did not change more than 2-fold after TNF stimulation. The mRNA level of the anti-apoptotic gene Bcl-x was diminished 4-fold by TNF but not by AGE. Two other pro-apoptotic genes, Nop30 and ATM, were down-regulated by AGE but not by TNF. A list of other apoptotic genes whose expression was not enhanced or diminished more than 2-fold is shown in Table III.

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involved and the downstream events. By use of a blocking antibody, it was shown that virtually all of the apoptotic effect of AGEs on fibroblast apoptosis was mediated through the RAGE. Previously, it was shown that RAGE activation reduces collagen production in fibroblasts (34). In addition, it has been reported that blocking RAGE significantly enhances wound healing in diabetes (35). As discussed below, enhanced fibroblast apoptosis represents one of the potential mechanisms through which AGEs could impair the healing process.

By using different caspase inhibitors in vivo and in vitro, we established that AGEs activate caspase-8, which signals apoptosis through the cytosolic pathway, and, to a lesser extent, caspase-9, which signals through the mitochondrial pathway. The functional importance of caspase activation in fibroblast apoptosis was shown by use of specific inhibitors. Consistent with the degree of activation, inhibition of caspase-8 in vitro had a greater impact than inhibition of caspase-9. However, caspase-8 and -9 inhibitors together were more potent than either inhibitor alone in reducing AGE-induced apoptosis, further supporting the concept that AGE signaling involves both caspase-8 and -9 pathways. Inhibitor studies also showed that both of these pathways stimulated activation of the effector caspase, caspase-3. The involvement of caspase-3 in AGE-stimulated fibroblast apoptosis was shown by enhanced caspase-3 activity and through inhibition of apoptosis with a specific caspase-3 inhibitor. The observation that caspase-3 inhibitor had an effect similar to that of a pan-caspase inhibitor suggests that AGE stimulation operates principally through this effector caspase. These results are in contrast to TNF, which induces fibroblast apoptosis almost exclusively through the cytosolic, caspase-8-dependent pathway (47).

To date, there have been relatively few reports examining the effect of AGEs on gene expression and no reports examining global induction of apoptotic genes. We demonstrated that AGEs induced a full range of apoptotic genes involving ligands, receptors, adaptor molecules, mitochondrial proteins, and various other molecules that participate in apoptosis. Although the net effect was to induce expression of pro-apoptotic genes, there were instances in which the expression of some was down-regulated, for example, the adapter molecules TRAF3, TRAF4, and TRAF5. For those genes whose expression has previously been reported to be induced by AGEs, we observed similar results, even though the cell type was different. For example, AGEs have been reported to down-regulate Bcl-2 in retinal pigment epithelial cell and pericytes (39, 53) and to up-regulate bax mRNA levels in mesangial cells and retinal neurons (54, 55). Similar down-regulation of bcl-2 and up-regulation of bax were found in AGE-stimulated fibroblasts.

The modulation of apoptotic gene expression was also compared between AGE and TNF-α stimulation under identical conditions. Whereas TNF induced a pattern that was overwhelmingly pro-apoptotic, including the inhibition of several anti-apoptotic genes, AGE stimulated a somewhat different pattern as discussed above. This would suggest that there are differences in the pathways through which AGE and TNF affect mRNA levels of apoptotic genes. A potential mechanism by which apoptosis through AGE or TNF could differ is through differences in stimulation of anti-apoptotic mechanisms. Although both AGEs and TNF have been shown to activate NF-κB (56, 57), it has also been reported that there are differences with AGEs exhibiting a more prolonged induction (58). Thus, AGEs and TNF may have differences in the pathways that lead to activation of caspase activity or may have differences in activation of NF-κB, thereby exhibiting differences in anti-apoptotic processes.

Advanced glycation end products are present at much higher levels in individuals with diabetes and in aged individuals. With increasing age, there is a reduction in fibroblast proliferation and life span, an increase in their apoptosis, and a diminished rate of dermal wound healing (59, 60). Fibroblasts play a leading role in wound healing, particularly in the skin. To participate in wound repair, they must migrate into the wounded area, proliferate, and form new matrix. Because adequate healing requires a sufficient number of cells to repair wounds, factors that cause enhanced apoptosis could impair healing, as suggested by recent reports (44, 61). Thus, the formation of advanced glycation end products represents one of the potential mechanisms through which wound healing in aged and diabetic individuals may be diminished.

Acknowledgment—We thank Alicia Ruff for help in preparing the manuscript.

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