PCNA could be due to a direct effect on PCNA or could be due to the induction of interferon. Interferons, and interferon-like substances, have been known to inhibit cell proliferation (*17*).

We cannot rule out this possibility even though it has not been shown that oligodeoxynucleotides induce interferon (*18*). PCNA is a cofactor of DNA polymerase δ, and it is believed that DNA polymerase δ is involved in cellular DNA replication (*19*). The finding that an antisense to PCNA results in complete suppression of DNA synthesis and of cellular proliferation indicates that PCNA is required for both cellular DNA synthesis and cell cycle progression. A reasonable amount of PCNA is still present in quiescent cells (*12*) or in cells inhibited by antisense oligodeoxynucleotides; the onset of cellular DNA synthesis may depend on a critical amount of PCNA. Finally, we would suggest that exposure of cells to antisense oligodeoxynucleotides may conveniently replace the more cumbersome use of antisense RNAs.

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20. Supported by grant CD-214 from the American Cancer Society (R.B.), grant CA 42866 from NIH (W.E.M.), and a grant from the Leukemia Research Foundation (B.C.).

22 February 1988; accepted 19 April 1988

Cachectin/TNF and IL-1 Induced by Glucose-Modified Proteins: Role in Normal Tissue Remodeling

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Proteins undergo a series of nonenzymatic reactions with glucose over time to form advanced glycosylation end products (AGEs). Macrophages have a receptor that recognizes the AGE moiety and mediates the uptake and degradation of AGE proteins. This removal process is associated with the production and secretion of cachectin (tumor necrosis factor) and interleukin-1, two cytokines with diverse and seemingly paradoxical biological activities. The localized release and action of these cytokines could account for the coordinated removal and replacement of senescent extracellular matrix components in normal tissue homeostasis.

MACROPHAGES PRODUCE THE POtent cytokine cachectin [also called tumor necrosis factor (TNF)] in response to bacteria, viruses, and parasitic organisms (*1*). This 17-kD protein was first identified as a factor able to promote hemorrhagic necrosis in some transplanted tumors (*2*) and kill several transformed cell lines (*3*). Soon thereafter, this same protein was independently isolated (*4*) and cloned (*5*) as a mediator of cachexia and shock. A number of studies with recombinant cachectin/TNF demonstrated the pluripotent effects of this protein. Included among these diverse bioactivities are enhancement of fibroblast growth (*6*), stimulation of collagenase release from several mesenchymal cell types (*7*), bone resorption accompanied by increased osteoclast and decreased osteoblast number (*8*), promotion of angiogenesis (*9*), and induction of a number of growth factors including granulocyte-macrophage colony-stimulating factor (*10*), platelet-derived growth factor (*11*), and interleukin-1 (IL-1) (*12, 13*), itself a known growth promoter and proteolytic enzyme inducer. The paradoxical capacity of these cytokines to promote both necrotic and growth responses in tissue suggest that cachectin/TNF and IL-1 might in fact serve as the mediators of a single important biological process—normal tissue remodeling. In addition to their role during invasion, monocyte-derived macrophages are believed to play an important role in tissue homeostasis in response to senescence or local injury by regulating mesenchymal cells and turnover of extracellular matrix proteins (*14*). Although effects of cachectin/TNF and IL-1 could account for several central features of tissue homeostasis, no endogenous stimulus regulating physiologic, noncytotoxic secretion of these mediators has yet been identified.

Recently, we identified a novel membrane-associated receptor on both peritoneal macrophages and human peripheral monocytes ($K_a = 10^7 M^{-1}$, 10^5 sites per cell) that specifically recognizes proteins modified by advanced glycosylation end products (AGEs) (*15, 16*). Using different proteins such as myelin proteins and low density lipoproteins, we showed that the AGE receptor recognizes only the AGE moiety, irrespective of type of protein (*15–17*). Proteins that are enzymatically glycosylated have carbohydrate structures totally unrelated to the nonenzymatically formed AGEs and do not bind to the AGE receptor. These irreversible nonenzymatic protein modifications form, through a series of slowly occurring dehydrations and rearrangements of the nonenzymatic addition product of glucose with protein amino groups, the Amadori product (*18, 19*). One of these adducts has been identified as 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (FFI), which forms from the condensation of two glucose molecules and two free lysine ε-amino groups of protein (*20*). Proteins with only Amadori-stage adducts are not recognized

Table 1. Detection of IL-1 production of human monocytes in response to AGE proteins. Normal human monocytes were prepared as described in Fig. 1. IL-1β was determined in total cell lysates by radioimmunoassay as described (*28*). Analysis of data ($n = 6$), shown here as means ± SD, by one-way analysis of variance (*32*), indicated that the difference between glucose-modified albumin (AGE-BSA) and normal unmodified albumin (Nl-BSA) is statistically significant ($P < 0.002$). All other P values were also <0.001.

Ligand added	IL-1β (p*M*)
AGE-BSA (250 μg/ml)	93.5 (± 29.9)
Nl-BSA (250 μg/ml)	16.2 (± 1.6)
IFN-γ (1 ng/ml)	None detected
LPS (0.2 ng/ml)	372.0 (± 36.4)

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by the macrophage AGE receptor (*17*). AGEs, unlike their dissociable precursors, continue to accumulate on slowly turning over tissue proteins for the life of the molecules. This accumulation results in the progressive formation of glucose-derived protein cross-links as a function of time (*21*) and serves as a biological marker of protein age. Thus, the interactions of an AGE protein with its receptor could provide a mechanism for selective targeting of macrophages to a time-dependent protein modification in vivo. We hypothesized that cross-linked senescent macromolecules would be preferentially removed by cytokine-dependent proteases and replaced by newly synthesized material if AGE recognition by macrophages is coupled to secretion of multifunctional cytokines such as cachectin/TNF and IL-1. In order to evaluate a key step of this hypothesis, we tested the effect of AGE proteins on cachectin/TNF and IL-1 secretion by macrophages in vitro.

We report that AGE proteins do induce macrophages to synthesize and release cachectin/TNF and IL-1. AGE proteins are thus the first reported endogenous nonpathological inducers of these cytokines. The paracrine effects of local cachectin/TNF secretion could account for the coordinated removal and replacement of senescent tissue proteins.

Freshly isolated human monocytes were incubated in endotoxin-free, polymyxin-treated medium with the macrophage primer interferon-γ (IFN-γ) (*22*) and one of the following: bovine serum albumin (BSA) (negative control); lipopolysaccharide (LPS) (positive control); albumin modified with AGEs formed naturally by incubation with glucose or glucose-6-phosphate (Glu-BSA, G-6-P-BSA), or formed synthetically (FFI-BSA) as described (*17*). IFN-γ was added in order to transform peripheral monocytes into the equivalent of primed tissue macrophages (*22, 23*). In these experiments IFN-γ was added at the same time as AGE protein, since preliminary data showed that simultaneous addition of IFN-γ gave the same results as a 6-hour pretreatment. Immunoreactive cachectin/TNF was detected with a sensitive enzyme-linked immunosorbent assay (ELISA) (*24*) (Fig. 1 legend).

Conditioned medium from macrophages incubated with BSA contained minimal levels of cachectin/TNF (Fig. 1). In contrast, conditioned medium from macrophages incubated with each of the three types of AGE-BSA contained approximately ten times the amount of cachectin/TNF found with normal (unmodified) BSA. Interferon-γ alone elicited no detectable cachectin/TNF production in identical incubations (*25*) (Fig. 1). In the absence of IFN-γ, human monocyte supernatants contained approximately one-half the amount of cachectin/TNF in response to AGE protein. This difference presumably reflects the ability of IFN-γ to exert a priming effect on human monocytes that augments their subsequent cachectin/TNF secretory response (*22, 23, 25*).

To determine whether the observed appearance of cachectin/TNF in conditioned media involved induction of new cachectin/TNF mRNA or translation of cryptic cachectin/TNF mRNA (*26*), we performed DNA blot analysis of monocyte mRNA for cachectin/TNF (Fig. 2). A small amount of cachectin/TNF mRNA was detected in monocyte preparations incubated with unmodified albumin (Fig. 2). In contrast, cachectin/TNF mRNA was increased in each monocyte preparation that was incubated with an AGE-modified albumin. These results indicate that the AGE proteins, like the potent bacterial stimulus LPS, increase both the cellular levels of cachectin/TNF mRNA and the amount of secreted cytokine. The relative contribution of transcriptional,

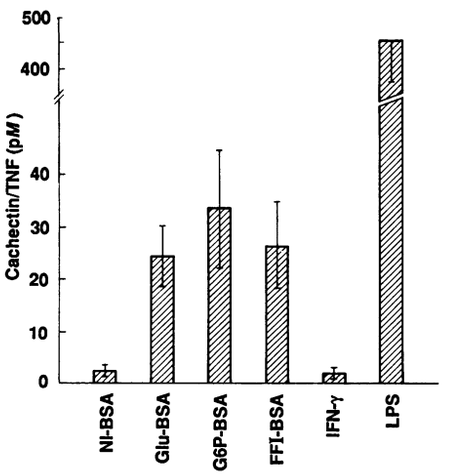

Fig. 1. Detection of cachectin/TNF secretion by human monocytes in response to AGE proteins. Normal human monocytes, isolated as described (*16*), were incubated in medium containing human IFN-γ (1 ng/ml, 50 U/ml) and polymyxin (100 ng/ml) in the presence of normal BSA (Nl-BSA, 250 μg/ml), BSA glycosylated in vitro (Glu-BSA, 250 μg/ml, or G-6-P-BSA, 250 μg/ml), or chemically synthesized AGE-BSA (FFI-BSA, 150 μg/ml) (*17, 20*), for 24 hours at 37°C. Additional control wells contained either nonstimulated cells, cells stimulated with LPS (0.2 ng/ml) or IFN-γ alone. All materials and media used were endotoxin free (<20 pg of LPS per milliliter) by a standard Limulus amebocyte lysate assay (*33*). After incubation, cachectin/TNF was measured in the media by ELISA with a purified monoclonal antibody to cachectin (SDW18.1.1, from a subclone of ATCC cell line HB9228 propagated as ascites tumors in CB6F1 mice; prepared by Chiron). This ELISA typically detects cachectin/TNF concentrations above 34 pg/ml (or 2 p*M*) (*24*). The low concentration (<5 p*M*) of immunoreactive cachectin/TNF detected in 24-hour supernatants of freshly isolated human peripheral monocytes incubated alone were subsequently subtracted from all other values. For each experimental condition, $n = 15$. Analysis of triplicate data by one-way analysis of variance (*31*) are shown as means ± SD. The difference between means of each of the modified BSA samples (Glu-BSA, G-6-P-BSA, FFI-BSA) and the unmodified Nl-BSA was statistically significant at $P < 0.002$.

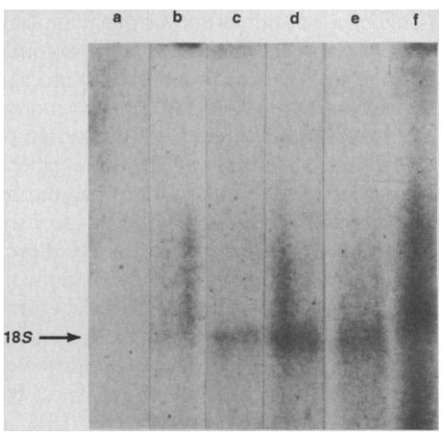

Fig. 2. Detection of cachectin/TNF mRNA from human peripheral blood monocytes by DNA blot analysis (*26*). Cells were isolated, plated, and incubated with ligands as described in the legend to Fig. 1. After media were removed for ELISA, cellular RNA (10×10^6 cells) was extracted, subjected to electrophoresis, blotted, and hybridized with radiolabeled cachectin/TNF probe as described (*26*). Cells were incubated with medium containing IFN-γ (1 ng/ml) throughout. (a) IFN-γ only, (b) unmodified BSA (250 μg/ml), (c) Glu-BSA (250 μg/ml), (d) G-6-P-BSA (250 μg/ml), (e) FFI-BSA (100 μg/ml), and (f) LPS (0.2 ng/ml). The position of the 18*S* ribosomal RNA band is indicated at the arrow.

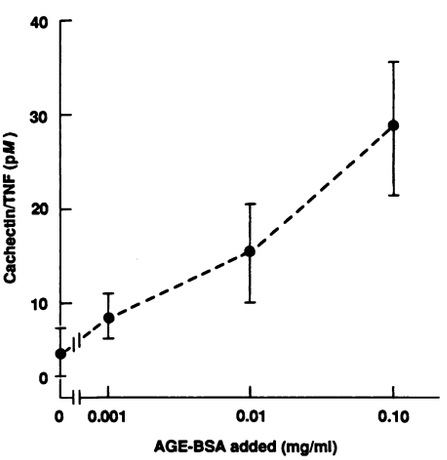

Fig. 3. Measurement of cachectin/TNF in the harvested media of normal human monocytes after a 24-hour incubation with different concentrations of FFI-BSA (0 to 100 μg/ml), as described in the legend to Fig. 1. Medium harvested from these incubations ($n = 3$) was subjected to the ELISA described in the legend to Fig. 1. Analysis of triplicate data, shown here as means ± SD, by one-way analysis of variance (*32*), indicated each mean value to be significantly different from each of the others ($P < 0.001$), except between point 0 and 1 μg/ml ($P < 0.08$).

translational, and posttranslational events to cachectin/TNF secretion induced by AGE proteins remains to be elucidated.

The effect of increasing concentrations of AGE-BSA (0 to 100 μg/ml) on the production of cachectin/TNF was investigated (Fig. 3). Generation of cachectin/TNF by monocytes was dependent on the amount of AGE-BSA added. Cachectin/TNF production increased linearly up to a maximum at a concentration of 100 μg/ml. This maximal stimulatory concentration is within the range found previously to saturate monocyte AGE-binding sites (*15*). The amount of AGEs added to each well in these experiments was of the same order of magnitude as that found in 50 μg of human coronary artery collagen. Thus the level of AGE protein used approximates that found in vivo.

The amount of cachectin/TNF elaborated after stimulation in vitro with AGE proteins is obviously much less than that induced in response to LPS. However, the excessive quantities of cytokine released by LPS in vivo are clearly pathological and result in acute cytotoxicity.

Additional data demonstrate that cell-associated, as well as extracellular IL-1 is also increased in response to AGE proteins under identical experimental conditions (Table 1). We measured total IL-1β produced in human monocytes after incubation with AGE protein, since membrane-bound IL-1 appears to account for a significant part of local stimulatory effects (*27, 28*). Even if its production represents a secondary response to cachectin/TNF, IL-1 still shares and would therefore amplify several of the above-mentioned activities of cachectin/TNF relating to tissue necrosis and remodeling (*29*).

These data demonstrate that AGE proteins can stimulate macrophages to secrete cachectin/TNF and IL-1 in noncytotoxic amounts. The presence of TNF in arterial wall and adjacent plaque areas suggest a role for this monokine in normal tissue remodeling (*30*). Under pathological circumstances, immune complexes could also contribute to localized secretion of monokines such as IL-1 (*31*). The mechanisms by which such cytokines could regulate tissue homeostasis are suggested by a variety of recent data. Previous studies have revealed that the degradative enzymes necessary for the removal of insoluble structural AGE proteins such as AGE-collagen from tissues are not themselves secreted by the macrophage (unpublished observations). We suggest instead that the recognition of AGE protein by macrophages induces the synthesis and secretion of TNF/cachectin and, directly or indirectly, IL-1; these cytokines in turn stimulate nearby mesenchymal cells to synthesize and release collagenase and other extracellular proteases (*6, 13*).

At the same time that these cytokines initiate local degradative events they could simultaneously stimulate other cells, such as fibroblasts (*6*), to undergo a synthetic/proliferative response. Cachectin/TNF and IL-1 have growth factor properties (*29*) and can stimulate the release of a number of other growth factors (*10, 11, 13*). Such a role for cachectin/TNF in normal tissue homeostasis may explain why this gene has been so highly conserved in mammals.

The in vivo modification of matrix proteins by time-dependent formation of glucose-derived AGEs may thus constitute a unique biologic time clock that signals macrophages to secrete cachectin/TNF, IL-1, and other cytokines, which in turn influence the degradation and proliferation of tissue components. An imbalance of this system in certain states, such as in diabetes where accelerated AGE formation occurs as a result of high glucose concentration (*18*), or in aging where AGE accumulation has increased as a function of time (*21*), may explain in part the excessive proliferative response characteristic of several diabetic and aging tissues.

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34. Supported in part by NIH grants RO1-AM19655, RO1-AM33861, and RO1-AI15674 and by grants from the Juvenile Diabetes Foundation and the Brookdale Foundation. We thank E. D. Spurgeon for excellent technical assistance.

16 November 1987; accepted 12 April 1988