157, 705 (1983); K. Haskins *et al.*, *ibid.*, p. 1149; L. E. Samelson, R. N. Germain, R. H. Schwartz, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6972 (1983).
2. P. Marrack and J. Kappler, *Adv. Immunol.* **38**, 1 (1986).
3. M. B. Brenner *et al.*, *Nature* **322**, 145 (1986).
4. A. M. Lew *et al.*, *Science* **234**, 1401 (1986).
5. A. Weiss, M. Newton, D. Crommie, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6998 (1986).
6. M. B. Brenner *et al.*, *Nature* **325**, 689 (1987).
7. D. Littman *et al.*, *ibid.* **326**, 85 (1987).
8. I. Bank *et al.*, *ibid.* **322**, 179 (1986); J. Borst *et al.*, *ibid.* **325**, 683 (1987); P. Moingeon *et al.*, *ibid.*, p. 723; B. Alarcon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3861 (1987); L. Lanier *et al.*, *J. Exp. Med.* **165**, 1076 (1987); S. L. Ang *et al.*, *ibid.*, p. 1453; N. Nakanishi, K. Maeda, K.-I. Ito, M. Heller, S. Tonegawa, *Nature* **325**, 720 (1987); D. M. Pardoll *et al.*, *ibid.* **326**, 79 (1987); F. Koning *et al.*, *Science* **236**, 834 (1987); L. A. Matis, R. Cron, J. A. Bluestone, *Nature* **330**, 262 (1987).
9. Y. Yanagi *et al.*, *Nature* **308**, 145 (1984); S. M. Hedrick, D. I. Cohen, E. A. Nielsen, M. M. Davis, *ibid.*, p. 153; H. Saito *et al.*, *ibid.* **309**, 757 (1984); Y. H. Chien *et al.*, *ibid.* **312**, 36 (1984); H. Saito *et al.*, *ibid.*, p. 31; G. K. Sim *et al.*, *ibid.*, p. 771.
10. Y. H. Chien, M. Iwashima, K. B. Kaplan, J. F. Elliott, M. M. Davis, *ibid.* **327**, 677 (1987).
11. S. Hata, M. B. Brenner, M. S. Krangel, *Science* **238**, 678 (1987).
12. E. Y. Loh *et al.*, *Nature* **330**, 569 (1987).
13. H. Band *et al.*, *Science* **238**, 682 (1987).
14. W. Born *et al.*, *Nature* **330**, 572 (1987); M. Bonyhadi, A. Weiss, P. W. Tucker, R. E. Tigelaar, J. P. Allison, *ibid.*, p. 574.
15. B. Toyonaga and T. Mak, *Annu. Rev. Immunol.* **5**, 585 (1987).
16. M. Kronenberg, G. Siu, L. E. Hood, N. Shastri, *ibid.* **4**, 529 (1986).
17. T. Quertermous *et al.*, *Nature* **322**, 184 (1986); M. P. Lefranc, A. Forster, R. Baer, M. A. Stinson, T. H. Rabbitts, *Cell* **45**, 237 (1986).
18. M. P. Lefranc, A. Forster, T. H. Rabbitts, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9596 (1986); T. Quertermous, W. M. Strauss, J. J. M. van Dongen, J. G. Seidman, *J. Immunol.* **138**, 2687 (1987); A. Forster, S. Huck, N. Ghanem, M. P. Lefranc, T. H. Rabbitts, *EMBO J.* 6, 1945 (1987); W. M. Strauss, T. Quertermous, J. G. Seidman, *Science* **237**, 1217 (1987).
19. F. Hochstenbach *et al.*, *J. Exp. Med.*, in press.
20. K. Satyanarayana, S. Hata, P. Devlin, J. L. Strominger, M. S. Krangel, in preparation.
21. H. Sakano, K. Huppi, G. Heinrich, S. Tonegawa, *ibid.* **280**, 288 (1979); P. Early *et al.*, *Cell* **19**, 981 (1980).
22. J. Kavaler, M. M. Davis, Y. H. Chien, *Nature* **310**, 421 (1984); G. Siu *et al.*, *ibid.* **311**, 344 (1984); S. P. Clark *et al.*, *ibid.*, p. 387.
23. F. Alt and D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4118 (1982).
24. Y. H. Chien *et al.*, *Nature 330*, 722 (1987).
25. M. S. Krangel, H. Spits, J. de Vries, unpublished results.
26. A. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983).
27. M. S. Krangel, H. Band, S. Hata, J. McLean, M. B. Brenner, *Science* **237**, 64 (1987).
28. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977); S. Tabor and C. C. Richardson, *ibid.* **84**, 4767 (1987).
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Inhibition of Cellular Proliferation by Antisense Oligodeoxynucleotides to PCNA Cyclin

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The proliferating cell nuclear antigen (PCNA or cyclin) is a nuclear protein recently identified as a cofactor of DNA polymerase δ. When exponentially growing Balb/c3T3 cells are exposed to antisense oligodeoxynucleotides to PCNA, both DNA synthesis and mitosis are completely suppressed. A corresponding sense oligodeoxynucleotide has no inhibitory effects. These experiments indicate that PCNA (cyclin) is important in cellular DNA synthesis and in cell cycle progression.

THE PCNA WAS ORIGINALLY described as a nuclear antigen detectable by immunofluorescence, and its appearance has been correlated to the proliferative state of the cell (*1*). A similar nuclear protein described by Bravo and co-workers was called cyclin (*2*). PCNA and cyclin were shown to be identical (*3*) and were identified as an auxiliary protein for DNA polymerase δ (*4*), necessary for the replication of simian virus 40 DNA (*5*). A partial-length cDNA clone of human PCNA was isolated and sequenced by Almendral *et al.* (*6*), and a full-length human cDNA clone was isolated (*7*) from an Okayama-Berg library (*8*).

We have constructed oligodeoxynucleotides complementary to either the sense or the antisense strand of the first 36 nucleotides of the PCNA sequence, beginning from the AUG codon.

The relevant PCNA sequence for these experiments includes the last 48 nucleotides that precede the AUG codon, as well as the first 72 nucleotides of the coding sequence (Fig. 1). Four 18-mer oligodeoxynucleotides were synthesized (Fig. 1). The first 18-mer (cyclin 1) extends from nucleotide 4 to nucleotide 21, inclusive. Cyclin 3 extends from nucleotide 22 to nucleotide 39 inclusive. Cyclin 2 and cyclin 4 are the antisense complementary strands of cyclin 1 and cyclin 3, respectively. The oligonucleotides were made on an Applied Biosystems 380B DNA synthesizer by means of β-cyanoethyl phosphoramidite chemistry. The overall yield of 18-mer was 80 to 85%, and the lyophilized product was redissolved in culture medium.

In these experiments we used Balb/c3T3 cells, exponentially growing on cover slips in the presence of Dulbecco's medium supplemented by 10% fetal bovine serum. The oligodeoxynucleotides [a mixture of cyclins 1 and 3 (sense) or cyclins 2 and 4 (antisense)] were added at time 0 to cultures without serum, as follows. The growth medium was removed, and the cultures were incubated in Dulbecco's medium (without serum) to which oligodeoxynucleotides (30 μ*M*) were added. After incubation for 30 min with oligodeoxynucleotides, serum was added to a final concentration of 10% without changing the medium. The cells were incubated for up to 24 hours, labeled with [^{3}H]thymidine (6.7 μCi/ml) for 30 min, and then fixed in methanol. Addition of antisense oligodeoxynucleotides markedly inhibited the proliferation of Balb/c3T3 cells. Cultures incubated with cyclins 2 and 4 remained sparse, whereas the corresponding controls and the cells exposed to sense oligodeoxynucleotides grew to 70% confluence, as evaluated by visual observation.

The labeling indices of these cultures, as measured after autoradiography, are shown in Fig. 2. We show two experiments in which the labeling index of the controls (no treatment) is slightly different, but these experiments have been repeated seven times and the results are reproducible. With this protocol, the labeling index of exponentially growing control cells should remain roughly constant, with time, as was observed (Fig. 2). Cells exposed to antisense oligodeoxynucleotides had a markedly decreased labeling index. The decrease began at 6 hours, and by 16 hours no labeled cells could be detected. In cells exposed to sense oligodeoxynucleotides the labeling index remained constant, at about the same level of their controls. Cells exposed to the sense oligodeoxynucleotides had a decrease in the number of autoradiographic grains per nucleus (20 to 30; the grains in the control could not be counted), although the percentage of labeled cells remained the same as in controls. The decrease is due, we believe, to the high amounts of sense and antisense oligodeoxynucleotides added to the cultures. It is conceivable that the oligodeoxynucleotides are

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slowly broken down, thus liberating enough thymidine triphosphate (dTTP) to considerably dilute the precursor pool. The amount added, 30 μM, is above the cellular concentration of dTTP, which is in the order of picomoles (*9*).

The effect of these oligodeoxynucleotides on cell proliferation, independent of the effect on the incorporation of [^{3}H]thymidine, was also measured by counting the number of mitoses per 1000 cells (Fig. 3). Addition of sense oligodeoxynucleotides to the cultures did not change the mitotic index, which remained indistinguishable from that of the controls (no treatment). However, in cells exposed to antisense oligodeoxynucleotides there was a complete suppression of mitosis and, in fact, no mitoses were observed in cells treated for 16 or 24 hours with antisense oligodeoxynucleotides.

In other experiments we used only one oligomer, the one extending from nucleotide 4 to nucleotide 21 inclusive (cyclins 1 and 2) and the results were identical. For example, 24 hours after addition of the oligodeoxynucleotides the labeling index of control cells was 47%, while that of cells exposed to the antisense 18-mer was <1%. The mitotic index was 1.6% in control cells, but no mitoses could be observed in the antisense treated cells. The addition of a single 18-mer antisense oligodeoxynucleotide sequence (whether it was cyclin 2 or cyclin 4) gave even more inhibition than the simultaneous addition of both 18-mers. The effect on cell proliferation was even more striking, with very little growth of the sparse cells, whereas control cells (with or without sense oligodeoxynucleotides) grew vigorously. A dose-response experiment with cyclin 2 showed that 2 μM had very little effect on the growth of Balb/c3T3 cells, 5μM gave ~50% inhibition, and 10 μM (or more) completely inhibited growth.

```
TTC CTC CTT CCC GCC TGC  CTG TAG

CGG CGT TGT TGC CAC TCC GCC  ACC

ATG TTC GAG GCG CGC CTG GTC  CAG

GGC TCC ATC CTC AAG AAG CTG  TTG

GAG GCA CTC AAG GAC CTC ATC  AAC
```

Fig. 1. Partial sequence of the PCNA gene. The coding strand includes 48 nucleotides preceding the ATG codon (the first triplet in the third row) and 72 nucleotides of the coding sequence. The synthesized 18-mer sequences are indicated by lines, either above or below the letters.

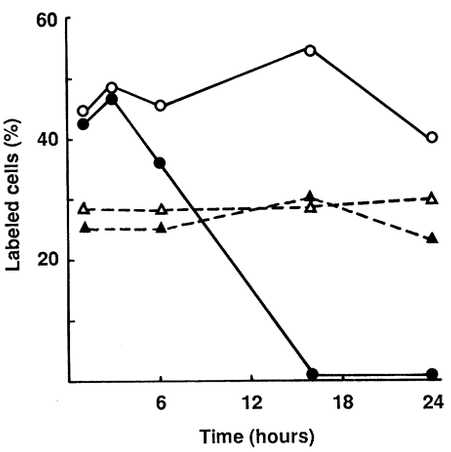

Fig. 2. Percentage of labeled Balb/c3T3 cells at various intervals after exposure to sense or antisense oligodeoxynucleotides. Cells were exponentially growing on cover slips and labeled with [^{3}H]thymidine for 30 min before fixation. ●, Cells exposed to antisense oligodeoxynucleotides; ○, their controls; ▲, cells exposed to sense oligodeoxynucleotides; △, their controls.

The amount of DNA per cell was measured by computer-operated microspectrofluorimeter in exponentially growing cells treated with cyclin 2 (*10*). Control cells or cells treated with a sense oligomer gave very close distributions: 48 to 50% of the cells had a G_1 DNA content, 40 to 42% had an S phase content, and 10% had a G_2 content. In cells treated with an antisense oligomer, G_2 cells completely disappeared, and the fraction of S phase cells tended to have lower DNA values. These data indicate that the block must occur at the G_1/S boundary or in the early S phase.

We also quantitated the expression of the PCNA protein in cells treated with antisense oligodeoxynucleotides. The amount of PCNA in control cells, and in cells exposed to either sense or antisense oligodeoxynucleotides, was determined by immunofluorescence (*11*) (Fig. 4). Cells treated with an antisense 18-mer (cyclin 2) for 24 hours show a marked decrease in the amount of nuclear PCNA. The amount of PCNA per cell was essentially the same in control cells (untreated) and in cells exposed to the sense 18-mer (Fig. 4, A and B). In cells exposed to the antisense 18-mer the amount of PCNA was about 40% that of the control cells (Fig. 4C). Bravo and Macdonald-Bravo (*12*) found that, after 24 hours of quiescence, the amount of PCNA (cyclin) was 30 to 40% that of growing cells. It seems, therefore, that the protein is relatively stable.

Reports have indicated that exposure of cells to antisense RNA to c-*fos* inhibits cell proliferation (*13*). Heikkila *et al.* (*14*) reported that human lymphocytes exposed to a 15-mer antisense oligodeoxynucleotide to c-*myc* do not enter the S phase after mitogen stimulation. Holt *et al.* (*15*) similarly found that an antisense oligodeoxynucleotide to c-*myc* inhibited cell proliferation and induced differentiation in HL-60 cells. The effect was not as dramatic as in our experiments, but we used much higher concentrations of oligomers, 30 μM versus 4 μM in their experiments. The inhibition of growth was reversible (*15*) and, as in our studies, removal of the antisense oligomer restored growth with a delay of about 36 to 48 hours (*16*).

We have shown that exposure of exponentially growing Balb/c3T3 cells to antisense oligodeoxynucleotides of PCNA completely inhibits cellular DNA synthesis and cellular proliferation. The inhibitory effect of the antisense oligodeoxynucleotides to

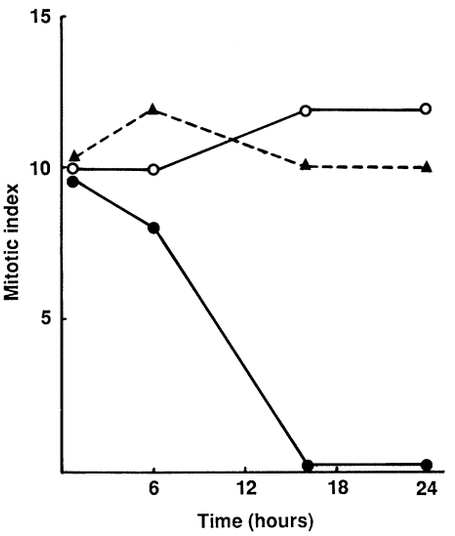

Fig. 3. Mitotic indices of Balb/c3T3 cells exposed to sense or antisense oligodeoxynucleotides. Number of mitoses per 1000 cells were determined at the indicated intervals. ●, Cells exposed to antisense oligodeoxynucleotides; ○, cells exposed to sense oligodeoxynucleotides; ▲, controls.

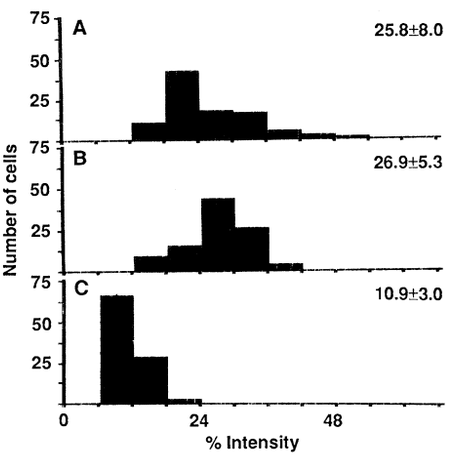

Fig. 4. Fluorescence intensity of Balb/c3T3 cells stained with a monoclonal antibody to PCNA. The abscissa is the percent fluorescence intensity as determined by a computer-operated microspectrofluorimeter. (**A**) Untreated cells; (**B**) cells treated with sense oligodeoxynucleotides; (**C**) cells treated with antisense oligodeoxynucleotides (n = 100 cells per graph).

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.

REFERENCES AND NOTES

1. K. Miyashi, M. J. Fritzler, E. M. Tan, *J. Immunol.* **121**, 2228 (1978).
2. R. Bravo, *Exp. Cell Res.* **163**, 287 (1986).
3. M. B. Mathews, R. M. Bernstein, B. R. Franza, Jr., J. I. Garrels, *Nature* **309**, 374 (1984).
4. R. Bravo, R. Frank, P. A. Blundell, H. Macdonald-Bravo, *ibid.* **326**, 515 (1987).
5. G. Prelich, M. Kostura, D. R. Marshak, M. B. Mathews, B. Stillman, *ibid.*, p. 471.
6. J. M. Almendral, D. Huebsch, P. A. Blundell, H. Macdonald-Bravo, R. Bravo, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1575 (1987).
7. D. Jaskulski *et al.*, *J. Biol. Chem.*, in press. This cDNA clone has 41 bp more than the one sequenced by Almendral *et al.* (*6*); the remaining sequences are identical.
8. H. Okayama and P. Berg, *Mol. Cell. Biol.* **3**, 280 (1983).
9. V. Bianchi, E. Pontis, P. Reichard, *ibid.* 7, 4218 (1987).
10. W. E. Mercer, C. Avignolo, R. Baserga, *ibid.* **4**, 276 (1984).
11. Cells were fixed in methanol and stained with an immunoglobulin G (IgG) monoclonal antibody to cyclin (ABT 151, American Biotech) at a 1:100 dilution. The secondary antibody was goat antibody to mouse IgG conjugated with fluorescein isothiocyanate (Cappel) at a 1:20 dilution.
12. R. Bravo and H. Macdonald-Bravo, *J. Cell Biol.* **105**, 1549 (1987).
13. J. T. Holt, T. V. Gopal, A. D. Moulton, A. W. Nienhuis, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4794 (1986).
14. R. Heikkila *et al.*, *Nature* **328**, 445 (1987).
15. J. T. Holt, R. L. Redner, A. W. Nienhuis, *Mol. Cell. Biol.* **8**, 963 (1988).
16. D. Jaskulski, J. K. deRiel, W. E. Mecer, B. Calabretta, R. Baserga, in preparation.
17. S. L. Lin, T. Kikuchi, W. J. Pledger, I. Tamm, *Science* **233**, 356 (1986).
18. P. Marcus, personal communication.
19. R. A. Hammond, J. J. Byrnes, M. R. Miller, *Biochemistry* **26**, 6817 (1987).

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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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