Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 259-265

IGF-1 RECEPTOR LEVELS AND THE PROLIFERATION OF YOUNG AND SENESCENT HUMAN FIBROBLASTS

CHRISTIAN SELL*, ANDRZEJ PTASZNIK**, CHUNG-DER CHANG*, JENNIFER SWANTEK*, VINCENT J. CRISTOFALO ** and RENATO BASERGA*†

*Jefferson Cancer Institute,
Thomas Jefferson University, Philadelphia, PA 19107

**Center for Gerontological Research,
Medical College of Pennsylvania, Philadelphia, PA 19129

Received May 28, 1993

We have investigated the role of the IGF-1 receptor in the proliferation of young and senescent human diploid fibroblasts. Using WI-38 cells, we have established the following : 1) both young and senescent cells have IGF-1 receptors, which can be autophosphorylated by IGF-1, the intensity of the autophosphorylation being roughly the same in both types of cells; 2) the levels of IGF-1 receptor mRNA are also similar in young and senescent cells; 3) both young and senescent cells have an absolute requirement for the IGF-1 receptor in order to be stimulated by either serum or SV40, respectively; 4) despite these similarities, young cells respond to IGF-1 (in combination with other growth factors) with DNA synthesis and mitosis, and senescent cells do not. We conclude that, although the IGF-1 receptor is still needed by senescent cells for a growth response to SV40, it is not, by itself, the determinant of senescence, at least in WI-38 cells.

© 1993 Academic Press, Inc.

IGF-1 is a growth factor required by several types of cells, including fibroblasts, epithelial cells, osteoblasts, chondrocytes, hemopoietic cells and others (1,2). In fibroblasts and fibroblast-like cells in culture, such as mouse 3T3 cells and WI-38 human diploid fibroblasts, IGF-1 is required for growth, together with at least one other growth factor, usually PDGF (3,4) or EGF (5). Other growth factors, for example FGF, can also complement IGF-1 (6). IGF-1, by itself, is non-mitogenic for quiescent fibroblasts (3,7,8), but requires, to stimulate growth, the previous or concomitant addition of the other growth factors (PDGF or EGF). Yet, the IGF-1 receptor is present in quiescent cells (8,9), and, when activated by its ligands, sends intracellular signals leading to the activation of specific genes (10-14). Furthermore, the time of entry into S phase is determined by the time of addition of IGF-1 (6,15), as if the cell cycle clock started with the activation of the IGF-1 receptor. In this paper, we have examined the importance of the IGF-1 receptor in the response of young and senescent human diploid fibroblasts to growth stimuli. For this purpose, we have used WI-38 cells, that are well characterized. It is well established that

† To whom correspondence should be addressed. Fax: (215) 923-0249.

0006-291X/93 $4.00
Copyright © 1993 by Academic Press, Inc.
All rights of reproduction in any form reserved.

young WI-38 cells respond to stimulation by growth factors (which include IGF-1), with cellular proliferation, while senescent cells fail to do so (16-18). Senescent WI-38 cells, however, can be induced to enter DNA synthesis by infection with SV40 (6,19). We therefore compared IGF-1 receptor levels in young and senescent cells and asked whether both serum-stimulated young cells and SV40-stimulated senescent cells required a functional IGF-1 receptor for their response.

MATERIALS AND METHODS

Cell Culture. WI-38 human diploid fibroblasts were grown in our laboratories as previously described (20,21). Young cells used in these experiments were at population doubling level (PDL) 25-26, while senescent cells were used at PDL 65 (22), at which point, the cells are still viable but do not grow any longer. Young cells were seeded in growth medium plus 10% fetal bovine serum at a concentration of $1x10^4/cm^2$ for all experiments, and were left undisturbed until stimulation with fresh medium supplemented with 10% fetal bovine serum or the indicated growth factors (see Results). Senescent cells were prepared as described (22,23) and otherwise treated like young cells.

SV40 Infection of Senescent Cells. A high titer lysate (10^7 cfu/ml) was used to infect senescent WI-38 cells at a high multiplicity of infection (10:1). Growth of SV40 virus, infection of cells and assay for DNA synthesis were carried out as previously described (19). Mock infected cells received only fresh growth medium.

Antisense Experiments. These were carried out with the modalities and with the same oligodeoxynucleotides described in previous papers from our laboratories (8, 24, 25). In the papers mentioned above, we have presented evidence that the antisense oligodeoxynucleotide to the IGF-1 receptor RNA (26) is specific for the targeted RNA and causes a marked decrease in the number of IGF-1 receptors. The oligomers were added in serum-free medium at a concentration of 40 µg/ml., unless otherwise noted.

Labeling Indices. For nuclear labeling, young cells were incubated with 3H thymidine, 1.0 µCi/ml for 24 hrs. After washing, the cells were fixed in cold methanol and processed for autoradiography.The procedure was similar for senescent cells infected with SV40 .

Autophosphorylation of the IGF-1 Receptor. This was carried out as previously described (25,27) on lysates of cells, using a monoclonal antibody to the IGF-1 receptor, ∂IR3 (Oncogene Sciences, Uniondale,N.Y.), an anti-phosphotyrosine antibody (UBI, Saranac Lake,N.Y.) and the ECL detection system from Amersham, according to the instructions of the kit's manufacturer.Protein concentrations were determined by the method of Bradford (28), and equal amounts of protein were added to each immunoprecipitation.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR). RNA was isolated by the method of Chomczynski and Sacchi (29). The RT-PCR was carried out essentially as described by Rappolee et al. (30). with the amplimers, probes, controls and modifications previously described from our laboratory (8, 25, 27). The amount of RNA was monitored in parallel reactions, by co-amplifying, with the IGF-1 receptor RNA, the RNA for ß2 microglobulin. The amplimers for the latter one derived from nucleotides 64-84 (TCA CGT CAT CCA GCA GAG AA) and 330-350 (GTA AGC AGC ATC ATG GAG GT) of the published sequence (31).

RESULTS

An Antisense Oligodeoxynucleotide to IGF-1 Receptor RNA Inhibits DNA Synthesis in both Young and Senescent WI-38 Cells.

In previous studies, we have shown that an antisense oligodeoxynucleotide to IGF-1 receptor RNA inhibits the growth of different cell types, such as 3T3 cells (8,27), human T

lymphocytes (24) and human prostate cancer cells (32). In those papers, we established that the antisense used was specific for the IGF-1 receptor RNA, and caused a decrease in the amount of autophosphorylated receptor varying from 60 to 80%. A typical experiment with this antisense oligodeoxynucleotide, Fig. 1, panel A, shows that it inhibits the growth response of young WI-38 cells to serum (measured as percentage of cells labeled by ^{3}H thymidine) by roughly 75%. The sense oligodeoxynucleotide also had some inhibitory effect, probably due to the toxic effects of the phosphorothioates, but clearly not as much as the antisense.

To study the effect of an antisense oligodeoxynucleotide to the IGF-1 receptor RNA on senescent cells, since senescent cells do not respond to serum, we infected them with SV40 (19), and determined the percentage of cells labeled by ^{3}H thymidine. Senescent cells are stimulated by

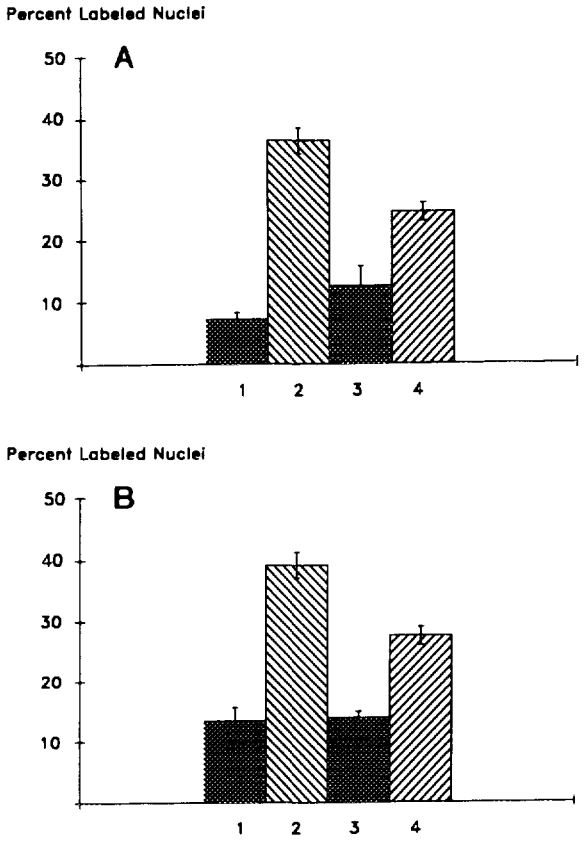

Fig. 1. Effect of an Antisense Oligodeoxynucleotide to the IGF-1 Receptor RNA on the Growth Response of WI-38 Cells.
The treatment is indicated on the abscissa; the ordinate gives the percentage of cells labeled by a 24 hr. exposure to ^{3}H thymidine. Panel A: percentage of labeled cells in young WI-38 cells stimulated with 5% serum in the presence or absence of oligodeoxynucleotides (40 µg/ml, added 24 hrs. before stimulation, see Methods and Materials). Panel B: percentage of labeled cells in senescent cells infected with SV40 (see Methods and Materials) also in the presence or absence of oligodeoxynucleotides.
Bars: 1, unstimulated controls; 2, serum or SV40-stimulated controls; 3, same as 2, plus addition of antisense oligodeoxynucleotides to the IGF-1 receptor RNA; 4, same as 2, plus sense oligodeoxynucleotides.

SV40 to enter DNA synthesis, but they do not divide (19). Fig.1, panel B, shows that SV40 infection causes an increase in the number of senescent WI-38 cells that enter S phase, an increase that is completely inhibited by an antisense (and much less by a sense) oligodeoxynucleotide to the IGF-1 receptor RNA. These experiments indicate that the IGF-1 receptor is required for the stimulation of quiescent young WI-38 cells by serum and of senescent cells by SV-40.

Serum and PDGF Increase the Levels of Autophosphorylated IGF-1 Receptor.

It has been known for some time that PDGF (or EGF) can increase the number of IGF-1 binding sites (33,34). It has also been reported that the number of IGF-1 binding sites is essentially similar in human diploid fibroblasts from young and aged donors (9). In the latter reference, however, the cells, although originating from aged donors, were not senescent and the contribution of IGF-binding proteins to the total binding had not been identified. Since autophosphorylation is a requirement for activation of the IGF-1 receptor and measures receptor activity (35,36), we asked whether serum or PDGF can actually increase the levels of IGF-1-dependent IGF-1 receptor autophosphorylation, in both young and senescent WI-38 cells. The results of an experiment with serum are shown in Fig.2. The ß subunit of the IGF-1 receptor is visible at roughly the 97 kd marker (non-specific bands can be recognized because they are not affected by IGF-1 stimulation, and were were present both above and below the ß subunit of the IGF-1 receptor). Panel A shows the effect of serum on quiescent young cells: in lane 1 is the cell lysate from unstimulated cells without IGF-1 (no receptor signal is visible, because the technique detects only a receptor autophosphorylated by its ligand); all the other lanes are lysates from cells incubated with IGF-1 for 15 min. prior to harvesting. The extent of IGF-1 receptor autophosphorylation increases after serum stimulation, especially between 4 and 16 hrs., decreasing a little at 24 hrs. post-stimulation (lanes 3 to 6). By densitometric analysis, the average of several experiments indicated that the amount of autophosphorylated IGF-1 receptor increased, 8 hrs. post-stimulation, 2.4-fold over unstimulated cells, with a range from 2.0 to 2.9-fold.

The levels of IGF-1 receptor autophosphorylation increase also in serum-stimulated senescent WI-38 cells (Fig.2, panel B); by densitometry, at peak values, the increase averaged 2.9 fold over unstimulated cells. Essentially the same results were obtained, in both young and senescent cells, when serum was replaced by PDGF stimulation (not shown). The increase, in both young and senescent cells, averaged 2.2 fold.

The IGF-1 Receptor RNA is Expressed in both Young and Senescent Cells.

We examined next the levels of IGF-1 receptor RNA in both young and senescent cells stimulated either with serum or PDGF. We show here an experiment with PDGF, but the same results were obtained with serum (not shown). Fig.3 shows that the levels of IGF-1 receptor RNA are essentially the same in young (panel A) and senescent (panel B) WI-38 cells. Indeed, if anything, it is increased in senescent cells. PDGF-stimulation causes only modest changes in receptor RNA levels, but in several experiments we noticed a consistent decrease at 24 hrs post-stimulation (lane 5 in both panels). The differences, at any rate, are not striking; the important point of Fig.3 is that IGF-1 receptor RNA levels are not decreased in senescent cells. This is the

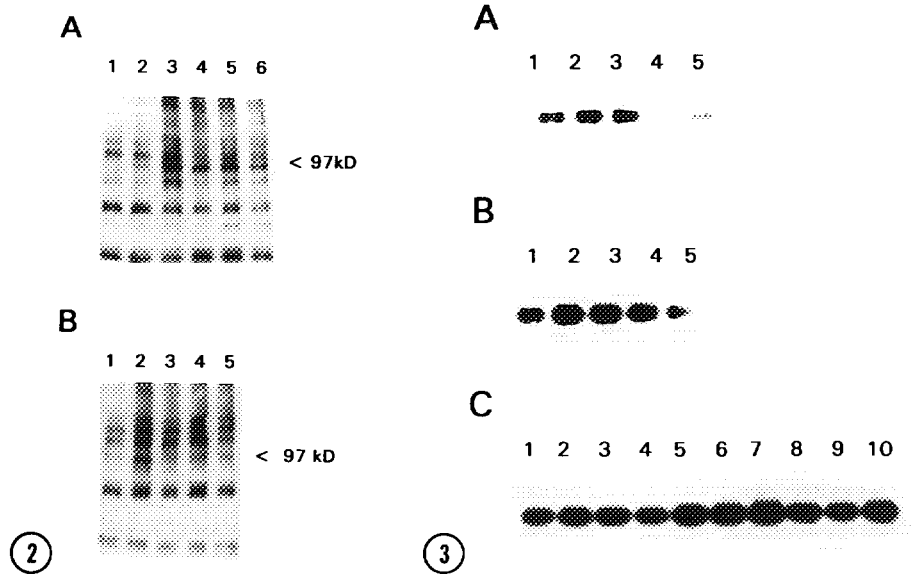

Fig. 2. Effect of Serum Stimulation on the Levels of Autophosphorylated IGF-1 Receptor in WI-38 Cells.
Lane 1 in panel A only is lysates from cells not pre-incubated with IGF-1; in all other lanes, the cells had been pre-incubated for 15 min. with IGF-1, prior to harvesting and lysis. The arrow indicates the autophosphorylated ß subunit of the IGF-1 receptor. Panel A: young cells, unstimulated (lane 2) and serum-stimulated for 4 hrs. (lane 3), 8 hrs. (lane 4), 16 hrs. (lane 5) and 24 hrs. (lane 6). Panel B: same experiment in senescent cells. Lanes 1-5 correspond to lanes 2-6 in panel A.

Fig. 3. Effect of PDGF Stimulation on the Levels of IGF-1 Receptor RNA in WI-38 Cells.
Panel A: young cells. Panel B: senescent cells. Lanes 1: unstimulated cells; lanes 2-5: 4, 8, 16 and 24 hrs. after stimulation of quiescent cells with PDGF. The levels of IGF-1 receptor RNA were determined by reverse transcriptase-polymerase chain reaction (see Methods and Materials). The amount of RNA per reaction was monitored in parallel reactions by co-amplifying the RNA for ß 2-microglobulin (31). The number of amplification cycles was 15 for the IGF-1 receptor RNA and 10 for ß 2-microglobulin.

opposite of what happens with IGF-1 RNA, which is present in substantial amounts in young cells but is absent in senescent cells (Ferber et al., submitted).

DISCUSSION

The novel findings in the present experiments are the following: 1) using the antisense strategy, we have shown that the IGF-1 receptor is required for the entry into S phase of serum-stimulated young WI-38 human diploid fibroblasts and SV-40 stimulated senescent cells. This is consistent with the many studies showing that IGF-1 is a required growth factor for many types of cells (1,2), and confirms our previous studies on the effect of antisense oligodeoxynucleotides to IGF-1 receptor RNA on other types of cells (8,24,25,27,34,37); 2) the IGF-1 receptor is present and can be autophosphorylated by its ligands in senescent human fibroblasts. Although a previous study had shown that IGF-1 binding sites were present in human diploid fibroblasts from aged donors (9), this is the first demonstration that the levels of IGF-1 receptor autophosphorylation are

essentially the same in young and senescent cells; and 3) the levels of IGF-1 receptor RNA are substantially similar in young and senescent cells.

Our results show that the IGF-1 receptor is required for a proliferative response of the young cells to growth factors and of senescent cells to SV40. However, cellular senescence in vitro cannot be simply explained by the requirement for a functional IGF-1 receptor. Senescent WI-38 cells have roughly the same level of autophosphorylated IGF-1 receptors as young cells, both before or after serum (or PDGF) stimulation, yet the cells do not respond to serum or IGF-1 with growth. Some other requirement must be postulated.

Finally, our studies complement a recent report by Moats-Staats, et al., (38) that an antisense oligodeoxynucleotide to the ligand, IGF-1, inhibits the growth of young WI-38 cells, in limiting serum conditions, again emphasizing the importance of the autocrine IGF-1/IGF-1 receptor loop in the growth of the young cells.

However, while our studies confirm the importance of the IGF-1 receptor activation in the proliferation of human diploid fibroblasts, other requirements are necessary to explain the behavior of senescent cells.

ACKNOWLEDGMENT

This work was supported by U.S. PHS grant AG 00378 from the National Institutes on Aging.

REFERENCES

1. Goldring, M.B., and Goldring, S.R. (1991) Eukar. Gene. Express. 1:301-326.
2. Lowe, W.L., Jr. (1991) In: Insulin-like growth factors: Molecular and cellular aspects (D LeRoith, ed). CRC Press, pp. 49-85.
3. Scher, C.D., Shephard, R.C., Antoniades, H.N. and Stiles, C.D. (1979) Biochem. Biophys. Acta 560:217-241.
4. Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, N.H., Van Wyk, J.J. and Pledger, W.J. (1979) Proc. Natl. Acad. Sci. USA 76:1279-1283.
5. Praeger, F.C. and Cristofalo, V.J. (1986) J. Cell. Physiol. 129:27-35.
6. Cristofalo, V.J., Phillips, P.D., Sorger, T. and Gerhard, G. (1989) J. Gerontol. 44:55-62.
7. Leof, E.B., Wharton, W., Van Wyk, J.J. and Pledger, W.J. (1982) Exp. Cell. Res. 141:107-115.
8. Pietrzkowski, Z., Lammers, R., Carpenter, G., Soderquist, A.M., Limardo, M., Phillips, P.D., Ullrich, A., and Baserga, R. (1992) Cell Growth & Diff. 3:199-205.
9. Clemmons, D.R., Elgin, R.G. and James, P.E. (1986) J. Clin. Endocrinol. Metab. 63:996-1001.
10. Surmacz, E., Kaczmarek, L., Ronning, O., and Baserga, R. (1987) Mol. Cell. Biol. 7:657-663.
11. Zumstein, P. and Stiles, C.D. (1987) J. Biol. Chem. 262:11252-11260.
12. Chiou, S.T. and Chang, W.C. (1992) Biochem. Biophys. Res. Comm. 183:524-531.
13. Damante, G., Cox, F. and Rapoport, B. (1988) Biochem. Biophys. Res. Comm. 151:1194-1199.
14. Santisteban, P., Acebron, A., Polycarpou-Schwartz, M. and DiLauro, R. (1992) Mol. Endocrin. 6:1310-1317.
15. Yoshinouchi, M. and Baserga, R. Cell Proliferation (in press).
16. Grove, G.L. and Cristofalo, V.J. (1977) J. Cell. Physiol. 90:415-422.
17. Rittling, S.R., Brooks, K.M., Cristofalo, V.J. and Baserga, R. (1986) Proc. Natl. Acad. Sci. 83:3316-3320.
18. Phillips, P.D., Pignolo, R.J. and Cristofalo, V.J. (1987) J. Cell. Phys. 133:135-143.

19. Gorman, S.D. and Cristofalo, V.J. (1985) J. Cell. Physiol. 125:122-126.
20. Cristofalo, V.J. and Charpentier, R. (1980) J. Tissue Culture Methods 6:117-121.
21. Chang, C.D., Phillips, P., Lipson, K.E., Cristofalo, V.J. and Baserga. R. (1991) J. Biol. Chem. 266: 8663-8666.
22. Cristofalo, V.J. and Sharf, B.B. (1973) Exp. Cell. Res. 76:419-427.
23. Phillips, P.D. Kaji, K. and Cristofalo, V.J. (1984) J. Gerontol. 39:11-17.
24. Reiss, K., Porcu, P., Sell, C., Pietrzkowski, Z. and Baserga, R. (1992) Oncogene 7:2243-2248.
25. Porcu, P., Ferber, A., Pietrzkowski, Z., Roberts, C.T., Adamo, M., LeRoith, D. and Baserga, R. (1992) Mol. Cell. Biol. 12:3883-3889.
26. Ullrich, A., Gray, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., LeBon, T., Katchuria, S., Chen, E., Jacobs, S., Francke, D., Ramachandran, J. and Fujita-Yamaguchi, Y. (1986) EMBO J. 5:2503-2512.
27. Pietrzkowski, Z., Sell, C., Lammers, R., Ullrich, A. and Baserga, R. (1992) Mol. Cell. Biol. 12:3883-3889.
28. Bradford, M.M. (1976) Anal. Biochem. 72:248-254.
29. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162:156-159.
30. Rappolee, D.A., Wang, A., Mark, D. and Werb, Z. (1989) J. Cell. Biochem. 39:1-11.
31. Suggs, S.V., Wallace, R.B., Hirose, T., Kawashima, E.H., Itakura, K. (1981) PNAS 78:6613-6617.
32. Pietrzkowski, Z., Mulholland, G., Gomella, L., Jameson, B.A., Wernicke, D. and Baserga, R. (1993) Cancer Res. 53: 1102-1106.
33. Clemmons, D.R. and Shaw, D.S. (1983) J. Cell. Physiol. 115-137-142.
34. Clemmons, D.R. and Van Wyk, J.J. (1981) In: Tissue Growth Factors. (R. Baserga, ed.), Springer-Verlag, KG, Berlin, pp. 161-208.
35. Kato, H., Faria, T.N., Stannard, B., Roberts, C.T., Jr. and LeRoith, D. (1993) J. Biol. Chem. 268:2655-2661.
36. Lammers, R., Van Obberghen, E., Ballotti, R., Schlessinger, J. and Ullrich, A. (1990) J. Biol. Chem. 265:16886-16890.
37. Pietrzkowski, Z., Wernicke, D., Porcu, P., Jameson, B.A. and Baserga, R. (1992) Cancer Res. 52: 6447-6451.
38. Moats-Staats, B.M., Retsch-Bogart, G.Z., Price, W.A., Jarvis, H.W., D'Ercole, A.J. and Stiles, A.D. (1993) Molec. Endo. 7: 171-180.