Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6309–6312, October 1982
Cell Biology

Microinjection of monoclonal antibody to protein p53 inhibits serum-induced DNA synthesis in 3T3 cells

(cell cycle/transformation/cell proliferation)

W. EDWARD MERCER*, DOUGLAS NELSON*, ALBERT B. DELEO†, LLOYD J. OLD†, AND RENATO BASERGA*

*Department of Pathology and Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140; and †Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10021

Contributed by Lloyd J. Old, July 22, 1982

ABSTRACT **Monoclonal antibody directed against the transformation-related protein p53 was microinjected manually into the nuclei of quiescent Swiss 3T3 mouse cells. The cells were subsequently stimulated with 10% fetal calf serum. Microinjection of p53 antibody at or around the time of serum stimulation clearly inhibited the subsequent entry of Swiss 3T3 cells into the S phase of the cell cycle. p53 antibody had no effect on serum-stimulated DNA synthesis when it was microinjected 4 hr or later after serum stimulation. Monoclonal antibody to an unrelated antigen, Lyt-2.2, had no effect on serum-stimulated DNA synthesis regardless of the time it was microinjected. Under similar experimental conditions, p53 antibody had no effect on simian virus 40- or adenovirus 2-induced DNA synthesis. These experiments add strength to the suggestion that p53 is involved in the regulation of cell proliferation.**

A protein with a molecular weight of approximately 53,000 (p53) has been identified in mouse cells transformed by viruses, chemicals, or x-radiation (1–6). This protein, although in smaller amounts, has also been detected in proliferating normal cells (7). Milner and Milner (8) reported that p53 is not synthesized in nondividing G_0 lymphocytes but is synthesized in the same lymphocytes when they are stimulated to proliferate by the addition of concanavalin A. Because p53 has been closely associated with cellular proliferation and transformation, it has been suggested that it is involved in the regulation of the mammalian cell cycle.

Several monoclonal antibodies have now been described that react with p53 of mouse cells by immunofluorescence and immunoprecipitation (5, 6, 9, 10). In the experiments reported here, we asked whether the microinjection of p53 monoclonal antibody into the nuclei of quiescent Swiss 3T3 cells would alter their proliferative response to serum stimulation.

MATERIALS AND METHODS

Cells. Swiss 3T3 cells obtained from Andrea Mastro (Pennsylvania State University) were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum. They were made quiescent by plating 2×10^5 cells in 60-mm Petri dishes containing a 22-mm^2 glass coverslip in medium containing 1% calf serum. The cultures were incubated at 37°C for 5 days. For stimulation of DNA synthesis, the medium was replaced with fresh Dulbecco's medium containing 10% fetal calf serum. SV3T3 was derived by transformation of Swiss 3T3 cells with simian virus 40 (SV40).

Antibodies. Two p53 monoclonal antibodies of mouse origin were used in these studies. One was a γ2a product of hybridoma 200-47, described by Dippold *et al.* (9), and the second was a γ2b product of hybridoma PAb 122, described by Gurney *et al.* (5). Lyt-2.2 monoclonal antibody (αLyt-2.2) was a γ2a product of hybridoma 19/178, originally derived by Ulrich Hämmerling. αLyt-2.2 was used as a microinjection control, because expression of Lyt-2.2 is restricted to cells of T-cell lineage. The reactivity of hamster antisera to the M_r 72,000 protein of adenovirus 2 (Ad2) has been described by Rossini *et al.* (11). Antisera having specificity for the SV40 T antigen were derived from hamsters bearing SV40 tumors (12).

Immunoglobulin Preparations. Immunoglobulin fractions were prepared by ammonium sulfate precipitation of (*i*) sera from *nu/nu* mice bearing hybridoma 200-47 (αp53) or hybridoma 19/178 (αLyt-2.2), (*ii*) supernatant fluid of cultures of hybridoma PAb 122 (αp53), or (*iii*) sera of hamsters bearing SV40-induced tumors (αSV40 T antigen). The precipitates obtained after the addition of 45% saturated ammonium sulfate were resuspended in phosphate-buffered saline and dialyzed extensively against the same buffer at 4°C prior to microinjection. In all cases, the concentration of microinjected protein was 2.5 mg/ml.

Microinjection Procedure. The microinjection procedure was carried out according to Graessmann and Graessmann (13) with slight modifications developed in our laboratory (12). Cells were microinjected at about 50% confluence. A circle was marked indelibly on the coverslip around a group of cells and the nuclei of all cells in the circle (142 to 270) were microinjected individually with the immunoglobulin fraction. The cells outside the circle were treated in the same manner, except for the microinjection, and served as controls.

SV40-Induced DNA Synthesis. DNA synthesis in quiescent Swiss 3T3 cells was induced by infection with purified SV40 virions at a multiplicity of infection of 100–500 plaque-forming units per cell. After 1 hr, the virus-containing medium was replaced with conditioned medium, and the infected cells were microinjected with the immunoglobulin fraction.

Ad2-Induced DNA Synthesis. DNA synthesis was induced in quiescent 3T3 cells by infection with Ad2 at a multiplicity of infection of 500 plaque-forming units per cell. Again, the infected cells were microinjected with the immunoglobulin fraction immediately after removal of the virus-containing medium.

Immunofluorescence Procedure. Mouse and hamster IgG were detected by staining with either fluorescein-conjugated goat anti-mouse IgG or goat anti-hamster IgG (Cappell Laboratories).

Autoradiography. After cultures were stimulated with 10% fetal calf serum, [^{3}H]thymidine (New England Nuclear; 6.7 Ci/mmol at 0.2 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) was added.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: SV40, simian virus 40; Ad2, adenovirus 2; α-, anti-; T antigen, SV40 tumor antigen.

Table 1. Effect of microinjected αp53 on serum-stimulated DNA synthesis in Swiss 3T3 cells

Microinjection			Labeled nuclei, %		
Ig	Time after stimulation, hr	Immunofluorescent nuclei, %	Microinjected cells	Control cells	Inhibition, %
αp53	−2	77.9	31.7	60.2	48
αLyt-2.2	−2	54.7	64.5	62.6	None
αp53	−0.5	63.1	23.0	50.7	55
αLyt-2.2	−0.5	73.0	47.7	50.8	None
αp53	+2	88.5	46.1	66.2	31
αLyt-2.2	+2	80.3	60.4	58.1	None
α53	+4	77.5	63.5	62.4	None
αLyt-2.2	+4	87.5	60.4	59.9	None
αp53	+6	73.6	45.1	46.5	None
αLyt-2.2	+6	62.5	53.2	50.7	None
αp53	+17	78.2	65.5	65.3	None
αLyt-2.2	+17	69.0	75.9	70.8	None

Swiss 3T3 cells were made quiescent and subsequently stimulated. The cells were microinjected with αp53 or αLyt-2.2 before or after serum stimulation, as indicated. All cells, microinjected or not, were labeled with [^{3}H]thymidine at 0.2 μCi/ml from the time of serum stimulation, with the exception of the cells microinjected 17 hr after serum stimulation. These cells were labeled for 0.5 hr with [^{3}H]thymidine at 10 μCi/ml. After labeling, the cells were stained with fluorescein-conjugated goat antibodies to mouse IgG; immunofluorescent nuclei are tabulated. Noninjected cells showed no fluorescence. Percentage of [^{3}H]thymidine-labeled cells was determined by autoradiography. Control cells were on the same coverslip as microinjected cells.

The cells were then fixed as indicated in *Results*, and autoradiographs were prepared and analyzed by standard methods.

RESULTS

Effect of the Microinjected p53 Monoclonal Antibody (αp53) on Serum-Induced DNA Synthesis. The results of two sets of experiments using αp53 produced by hybridoma 200-47 are summarized in Table 1. In the first set of experiments, nuclei of quiescent Swiss 3T3 cells were microinjected with αp53 or αLyt-2.2, stimulated with 10% fetal calf serum, and then labeled with [^{3}H]thymidine for 17 hr. In a second set of experiments, quiescent Swiss 3T3 cells were first stimulated with 10% fetal calf serum and then microinjected with αp53 at 2, 4, 6, or 17 hr after serum stimulation. Labeling with [^{3}H]thymidine was initiated after serum stimulation and continued for 17 hr, except in the case of cells injected at 17 hr, for which labeling was for 0.5 hr. The first experiment was designed to determine whether the αp53 could inhibit serum-stimulated DNA synthesis during the prereplicative phase. The second experiment was designed to determine two things: first, whether αp53 could inhibit DNA synthesis when microinjected after the addition of serum—i.e., during the G_1 phase—and second, whether αp53 microinjected at 17 hr after serum stimulation, when most cells were in S phase, could inhibit ongoing DNA synthesis.

The results given in Table 1 indicate that (*i*) microinjection of αLyt-2.2 has no effect whatsoever on serum-stimulated DNA synthesis or ongoing DNA synthesis in Swiss 3T3 cells and (*ii*) αp53 clearly inhibits serum-stimulated DNA synthesis when it is microinjected at, or around, the time of stimulation. When αp53 is microinjected 4 or 6 hr after serum stimulation, it has no inhibitory effect on serum-stimulated DNA synthesis. When microinjected when the cells are already in S phase, αp53 has no effect on ongoing DNA synthesis. These experiments were repeated four times. In all cases, when cells were microinjected with αp53 at 2 hr before to 2 hr after serum stimulation, inhibition of DNA synthesis was observed, although the degree of inhibition varied from one experiment to another. Table 1 also gives the percentage of cells that were immunofluorescent when microinjected with either αp53 or αLyt-2.2. This percentage reflects the percentage of cells that were still immunofluorescent for the injected immunoglobulin 17 hr after microinjection. Fig. 1 is a photomicrograph of Swiss 3T3 cells stained with fluorescein-conjugated goat anti-mouse IgG at 4

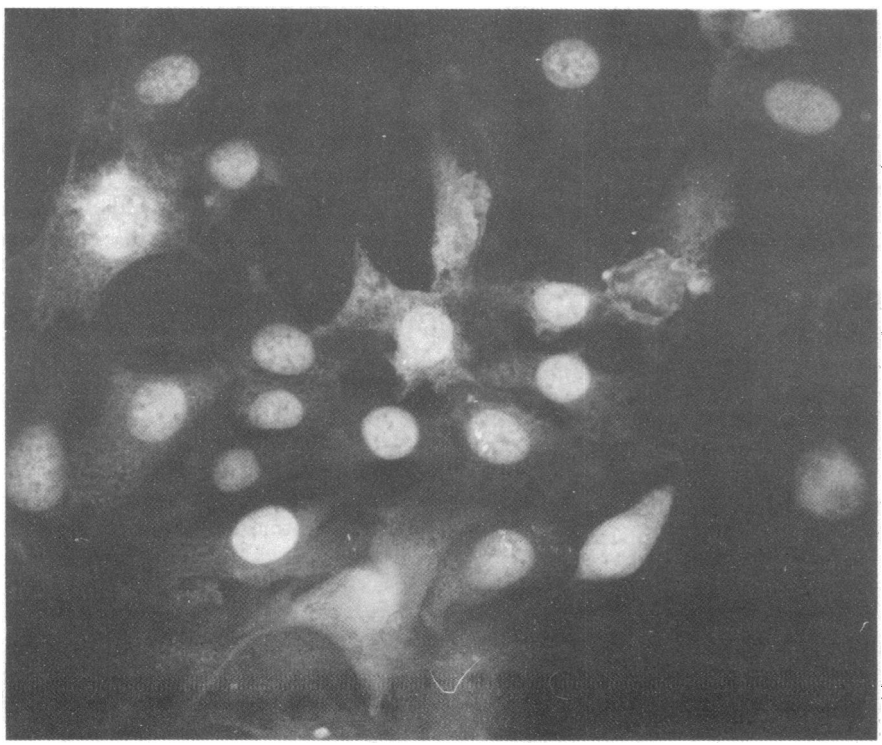

FIG. 1. Swiss 3T3 cells stained with fluorescein-conjugated goat anti-mouse IgG 4 hr after microinjection of αp53. (×250.)

hr after microinjection of αp53. A small number of cells show both cytoplasmic and nuclear staining; however, most of the microinjected αp53 remains localized within the nuclei of the injected target cells. Control cells outside the area of microinjection are totally negative for immunoglobulin. It should be noted that αp53-microinjected cells stain very brightly in the first few hours after microinjection but stain increasingly weaker with time and after 20 hr show little or no fluorescence. In contrast, staining of αLyt-2.2-injected cells remains bright and easily detectable, even at 24 hr after microinjection.

The same results were obtained with αp53 produced by hybridoma PAb 122. When this αp53 was microinjected 0.5 hr before the addition of serum, it inhibited serum-stimulated DNA synthesis to a level similar to that obtained with αp53 produced by hybridoma 200-47.

Effect of Microinjected αp53 on SV40- or Ad2-Induced Cellular DNA Synthesis. Table 2 shows the effect of microinjection with αp53 or αLyt-2.2 on cellular DNA synthesis induced by SV40 or Ad2 infection of quiescent 3T3 cells. Contrary to our expectations, the microinjected αp53 did not inhibit cellular DNA synthesis induced by either SV40 or Ad2. As can be seen in Table 2, infection of quiescent Swiss 3T3 cells with SV40 stimulated DNA synthesis in 42–47% of the cells, and Ad2 stimulated DNA synthesis in about 50% of the cells. Microinjection of αp53 or αLyt-2.2 had no effect on the number of cells entering S phase as a result of viral infection. However, antibody against SV40 T antigen clearly inhibited SV40-induced cell DNA synthesis, as already described by Floros *et al.* (12). With regard to the SV40-transformed cell line, SV3T3, Table 3 shows that cellular DNA synthesis was not inhibited by αp53.

Detection of p53 in Swiss 3T3 Cells and SV3T3 by Immunofluorescence. Quiescent, serum-stimulated, or virus-infected 3T3 cells were allowed to react with αp53 and then with fluorescein-conjugated goat anti-mouse IgG. With this indirect immunofluorescence method, p53 was not detectable in quiescent or serum-stimulated S-phase Swiss 3T3 cells, as already reported by Dippold *et al.* (9). However, at 32 hr after infection of quiescent 3T3 cells with SV40, both SV40 T antigen and p53 could be detected. In contrast, p53 could not be detected in Ad2-infected quiescent cells that were clearly positive for the Ad2 M_r 72,000 protein. In SV3T3 cells both SV40 T antigen and p53 were readily detectable by indirect immunofluorescence, regardless of whether the cells were grown in medium containing 1% calf serum or 10% fetal calf serum.

Table 2. Effect of microinjected αp53 on SV40- or Ad2-induced cellular DNA synthesis

Ig microinjected	Immunofluorescent nuclei, %	Labeled nuclei, %	
		Microinjected cells	Control cells
Experiment 1: SV40-induced			
αp53	64.4	43.7	47.6
αSV40 T antigen	70.2	16.2	45.9
αLyt-2.2	51.4	45.0	42.3
None, uninfected cells	—	—	1.0
Experiment 2: Ad2-induced			
αp53	65.0	50.1	52.0
αLyt-2.2	49.5	53.8	51.0
None, uninfected cells	—	—	1.2

Swiss 3T3 cells were made quiescent and infected with SV40 at 100–500 plaque-forming units per cell or Ad2 at 500 plaque-forming units per cell and after 1 hr of adsorption the original plating medium containing 1% calf serum was added to the cells. At 0.5 hr after addition of the medium, the cells were microinjected with αp53 or αLyt-2.2. The cells were labeled for 32 hr with [^{3}H]thymidine at 0.2 μCi/ml. After labeling, cells were stained for microinjected IgG as described in the legend to Table 1 and for the presence of SV40 T antigen or Ad2 M_r 72,000 protein by using hamster antiserum. The percentage of SV40 T antigen-positive cells in experiment 1 was 42.1% and the percentage of Ad2 M_r 72,000 protein-positive cells in experiment 2 was 35.0%. After indirect immunofluorescence staining, cells were processed for autoradiography.

Table 3. Effect of microinjected αp53 on DNA synthesis in SV40-transformed mouse cells

Ig microinjected	Immunofluorescent nuclei, %	Labeled cells, %	
		Microinjected cells	Control cells
αp53	77.7	94.2	97.8
αLyt-2.2	43.3	96.2	92.4

SV40-transformed cells (SV3T3) were maintained for 5 days in 1% calf serum. They were microinjected, shifted to 10% fetal calf serum, and labeled continuously for 20 hr with [^{3}H]thymidine at 0.2 μCi/ml.

DISCUSSION

It has been reported that antibodies microinjected into cells preserve their specificity of action without apparent toxicity (12, 14–16). Floros *et al.* (12) showed that preimmune IgG microinjected into the nuclei of quiescent 3T3 cells does not inhibit serum-stimulated DNA synthesis, whereas monoclonal antibody against the SV40 T antigen did specifically inhibit SV40-induced, but not serum-stimulated, DNA synthesis. These microinjected antibodies have a half-life of approximately 20 hr (14, 15). Using this technique, we have now determined that monoclonal antibody directed against p53 effectively inhibits serum-induced DNA synthesis when it is microinjected into the nuclei of quiescent cells at or around the time of serum stimulation. This effect cannot be attributed to toxicity because αp53 has no effect on serum-stimulated DNA synthesis when it is microinjected 4 hr after serum stimulation, and monoclonal antibody to an unrelated antigen, Lyt-2.2, has no effect on DNA synthesis of Swiss 3T3 cells regardless of when it is microinjected into the nuclei. Furthermore, when antibody against RNA polymerase I is microinjected into the nuclei of quiescent cells, serum-stimulated DNA synthesis is not inhibited at all, although nucleolar RNA synthesis is inhibited for several hours (unpublished data). Our results therefore strongly support the possibility proposed by Milner and Milner (8) and Jay *et al.* (7) that p53 is involved in the regulation of cell proliferation. These experiments, while not defining the role of p53 in the initiation of cell proliferation, do suggest that p53 is a key factor in the transition of cells from a resting to a growing stage. As to the reason why the microinjected antibody is effective only between −2 and +2 hr of stimulation, three possibilities can be raised: (*i*) p53 is no longer needed after the first few hours after stimulation; (*ii*) the amount of p53 that accumulates by 4 hr is greater than the binding capacity of microinjected αp53; or (*iii*) in view of the reported binding of p53 to DNA (17), bound p53 becomes inaccessible to antibody with time. Under similar experimental conditions, αp53 does not inhibit Ad2- or SV40-induced cell DNA synthesis. These results, particularly in the case of SV40, are surprising. However, because SV40 can overcome the block of G_1-specific temperature-sensitive mutants of the cell cycle (whereas serum stimulation cannot) (12), it is possible that SV40 T antigen may induce cell DNA synthesis independently of p53.

Novi and Baserga (18) reported that a short inhibition of protein synthesis by cycloheximide, for 2 hr, when carried out very early after stimulation of cell proliferation, completely inhibited the subsequent entry of cells into S phase. Similar doses of cy-

cloheximide were totally ineffective after the first few hours after stimulation. On the basis of these results, Novi and Baserga (18) speculated that a very labile gene product was made in the first 3–4 hr after stimulation and that if the synthesis of this product was inhibited, the whole process of stimulated DNA synthesis would come to a halt. This idea was reproposed in 1979 by Rossow *et al.* (19) and, more recently, by Pledger *et al.* (20) and Smith and Stiles (21). We suggest that this labile gene product, which is necessary for stimulation of cell proliferation and has a very short half-life in the beginning of the prereplicative phase, is p53.

This work was supported by National Cancer Institute Grants CA-12923 and CA-08373 to R.B. and CA-28461 to A.B.D.

1. DeLeo, A. B., Jay, G., Appella, E., DuBois, G. C., Law, L. W. & Old, L. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2420–2424.
2. Lane, D. P. & Crawford, L. V. (1979) *Nature (London)* **278**, 261–263.
3. Linzer, D. I. & Levine, A. J. (1979) *Cell* **17**, 43–52.
4. Melero, J. A., Tur, S. & Carroll, R. B. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 97–101.
5. Gurney, E. G., Harrison, R. O. & Fennell, J. (1980) *J. Virol.* **34**, 752–763.
6. Rotter, V., Witte, O. N., Coffman, R. & Baltimore, D. (1980) *J. Virol.* **36**, 547–555.
7. Jay, G., DeLeo, A. B., Appella, E., DuBois, G. C., Law, L. W., Khoury, G. & Old, L. J. (1980) *Cold Spring Harbor Symp. Quant. Biol.* **44**, 659–664.
8. Milner, J. & Milner, S. (1981) *Virology* **112**, 785–788.
9. Dippold, W. G., Jay, G., DeLeo, A. B., Khoury, G. & Old, L. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1695–1699.
10. Harlow, E., Crawford, L. V., Pim, D. C. & Williamson, N. M. (1981) *J. Virol.* **39**, 861–869.
11. Rossini, M., Jonak, G. J. & Baserga, R. (1981) *J. Virol.* **38**, 982–986.
12. Floros, J., Jonak, G., Galanti, N. & Baserga, R. (1981) *Exp. Cell Res.* **132**, 215–223.
13. Graessmann, M. & Graessmann, A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 366–370.
14. Yamaizumi, M., Uchida, T., Mekada, E. & Okada, Y. (1979) *Cell* **18**, 1009–1014.
15. Zavortink, M., Tacher, T. & Rechsteiner, M. (1979) *J. Cell. Physiol.* **100**, 185–186.
16. Antman, K. H. & Livingston, D. (1980) *Cell* **19**, 627–635.
17. Luka, J., Jornvall, H. & Klein, G. (1980) *J. Virol.* **35**, 592–602.
18. Novi, A. & Baserga, R. (1972) *Lab. Invest.* **26**, 540–547.
19. Rossow, P. W., Riddle, V. G. H. & Pardee, A. B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4446–4450.
20. Pledger, W. J., Hart, C. A., Locatell, K. L. & Scher, C. D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4358–4362.
21. Smith, J. C. & Stiles, C. D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4363–4367.