Decreased Sensitivity of Transformed 3T3-SV40 Cells Treated with N-Acetylcysteine to Bacterial Invasion

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Long-term treatment of transformed 3T3-SV40 mouse fibroblasts with antioxidant N-acetylcysteine decreased cell level of ROS and increased the concentration of reduced glutathione. Removal of N-acetylcysteine from the medium led to the appearance of well-expressed stress fibrils, virtually absent in control cells. In contrast to control cells, these cells were not invaded by apathogenic *Escherichia coli* A2 strain producing ECP32 protease specifically cleaving actin. Antioxidant N-acetylcysteine can cause partial reversion of transformed phenotype at the expense of a shift of cell redox balance in favor of reduced glutathione.

Key Words: *transformed 3T3-SV40 cells; active oxygen forms; glutathione; cytoskeleton; invasion*

A concept according to which antioxidants protect the cell and the body from the destructive effect of the oxidant excess is based on the data on the key role of ROS and oxidative stress in the pathogenesis of many human diseases. Antioxidant N-acetylcysteine (NAC), an L-cysteine derivative and reduced glutathione precursor (GSH) in the cell, is widely used in medicine. NAC is used in the therapy of many diseases, *e.g.* acute pancreatitis [14], chronic obstructive bronchitis [4], chronic renal diseases [11], in some acute poisonings [7]. However, the mechanisms underlying the effects of ROS and antioxidants (including NAC) remain unclear. Wide use of antioxidants with therapeutic purposes prompts investigation of morphological and functional changes in cells under conditions of balance shift towards antioxidants.

A shift of the cell redox balance towards ROS can play a key role in tumor transformation of normal cells. Among numerous morphological changes in cells under these conditions are reorganization of the cytoskeleton structures, including loss of microfilament bundles and changes in the system of focal contacts of cells with the substrate. Increased cell sensitivity to bacterial invasion (e.g. *Listeria monocytogenes* [12] or *E. coli* strain A2 [5] invasion) is a possible functional disorder resulting from cell transformation. Moreover, multiplication of bacteria is possible only in transformed cells [13]. Reorganization of the cytoskeleton structures during transformation is also referred to the causes of increased invasion of transformed cells. The mechanisms of penetration depend on pathogen species, but in any case the cortical (microtubular and actin) cytoskeleton is involved, while the actin cytoskeleton has to be reorganized for intracellular existence of the bacterium, though the molecular mechanisms of this reorganization can be different [3].

Recently we found that NAC antioxidant can modify (normalize) organization of actin cytoskeleton in SV40 virus-transformed 3T3 mouse fibro-

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blasts [2]. Actin microfilaments disassembled in the presence of NAC in 3T3-SV40 fibroblasts were restored after removal of NAC from the culture medium; well-expressed stress fibrils, virtually absent in control cells, but intrinsic of various normal cells, including immortalized 3T3 [2], emerged in the cells. In contrast to 3T3-SV40 cells, 3T3 cells are not invaded by apathogenic *E. coli* A2 strain producing ECT32 protease specifically cleaving actin [5].

Therefore we studied the sensitivity of transformed 3T3-SV40 fibroblasts with modified actin cytoskeleton as a result of NAC treatment to *E. coli* A2 bacterial invasion. The content of ROS and GSH concentration were measured in the same cells.

MATERIALS AND METHODS

Mouse (Balb/3T3) fetal fibroblasts transformed with SV40 virus (3T3-SV40 cell strain) were obtained from cell culture collection of Institute of Cytology. The cells were cultured in DMEM with 10% FCS until confluence. Antioxidant NAC (Sigma) was added to the culture medium to the final concentration of 10 mM for 20 h, after which the culture medium was replaced with a fresh portion without NAC and the cells were cultured for 24 h. Cells not treated with NAC served as the control.

For visualization of actin cytoskeleton elements, the cells were washed with phosphate buffered saline, fixed in 3.7% formalin for 10 min, treated with 0.1% Triton X-100 for 10 min, and stained with phalloidin-TRITC (Sigma) for 15 min at 37°C. The preparations were examined under an Axioscop microscope (×150); fluorescence was excited and recorded at λ=540 and 590 nm, respectively.

E. coli A2 strain producing ECP32 protease specifically cleaving actin [6] was used for invasion. The bacteria were cultured in LB medium at 37°C for 26 h. Bacterial suspension in growth medium was centrifuged at 67*g* for 20 min. The precipitate was resuspended in DMEM and added to the cells after medium change. The cells and bacteria were cocultured for 2 h at 37°C and 5% CO_2.

For electron microscopy, the cell precipitate after centrifugation in growth medium was fixed with 2% glutaraldehyde for 40-60 min at ambient temperature, washed in phosphate buffer, and post-fixed with 1% osmic acid for 30 min at ambient temperature. The cells were embedded in epon-araldite, ultrathin sections were contrasted with 2% uranyl acetate in 50% ethanol and with lead citrate and examined under a JEM-100C electron microscope at 80 kV.

Intracellular ROS formation was evaluated by the fluorescence intensity of ROS-dependent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) [1] at the probe concentration of 4 μM. Fluorescence was excited at λ=490 nm and recorded at 520 nm. The cells on slides were incubated with H_2DCF-DA for 5 min, after which the fluorescence intensity was measured in Hanks' solution (pH 7.4) at 37°C and ×100.

The concentration of GSH in the cells was evaluated colorimetrically by changes in optical density at λ=565 nm. The method is based on GSH displacement of aminasine from a colored complex with palladium chloride in acid medium [9].

RESULTS

Like other transformed cells, 3T3-SV40 cells have no clear-cut stress fibrils, characteristic of normal fibroblasts and other cells (Fig. 1, *a*). Culturing for 20 h in a medium containing 10 mM NAC led to virtually complete disassembly of the actin cytoskeleton and changes in cell shape (they became round); microphotographs revealed accumulation of amorphous actin (Fig. 1, *b*). The degree of changes in the cells varied, but the sum of observations indicated that the majority of cells undergo changes shown in Fig. 1, *a*. Longer treatment with NAC or increase of its concentration induced apoptotic cell death [1]. Removal of NAC from the culture medium 20 h after its addition led to recovery of cytoskeleton structure, although the restored cytoskeleton differed from the control (Fig. 1, *b*). Well-structured stress fibrils not observed in control 3T3-SV40 cells appeared in NAC-treated cells. By the shape, number of contacts, flattening, and presence of stress fibrils the cells resembled 3T3 fibroblasts rather than control 3T3-SV40 cells [1,6]. The degree of microfilament recovery varied in the cells of the same preparation; there were cells with destroyed actin cytoskeleton, but the overwhelming majority of cells had structured stress fibrils.

Since ROS and antioxidants attacking the actin thiol groups [6] can reorganize actin filaments, we wondered whether the observed changes were related to changes in intracellular ROS and GSH levels

TABLE 1. Content of ROS and Concentration of GSH in 3T3-SV40 Cells in the Presence of 10 mM NAC and after Its Removal from Culture Medium (*M*±*m*)

Experiment conditions	ROS, arb. units (*n*=5-7)	GSH, mM (*n*=3-5)
Control	11.5±0.8	0.45±0.03
+NAC, after 20 h	4.8±0.3	0.72±0.02
-NAC, after 20 h	7.0±0.5	0.67±0.03

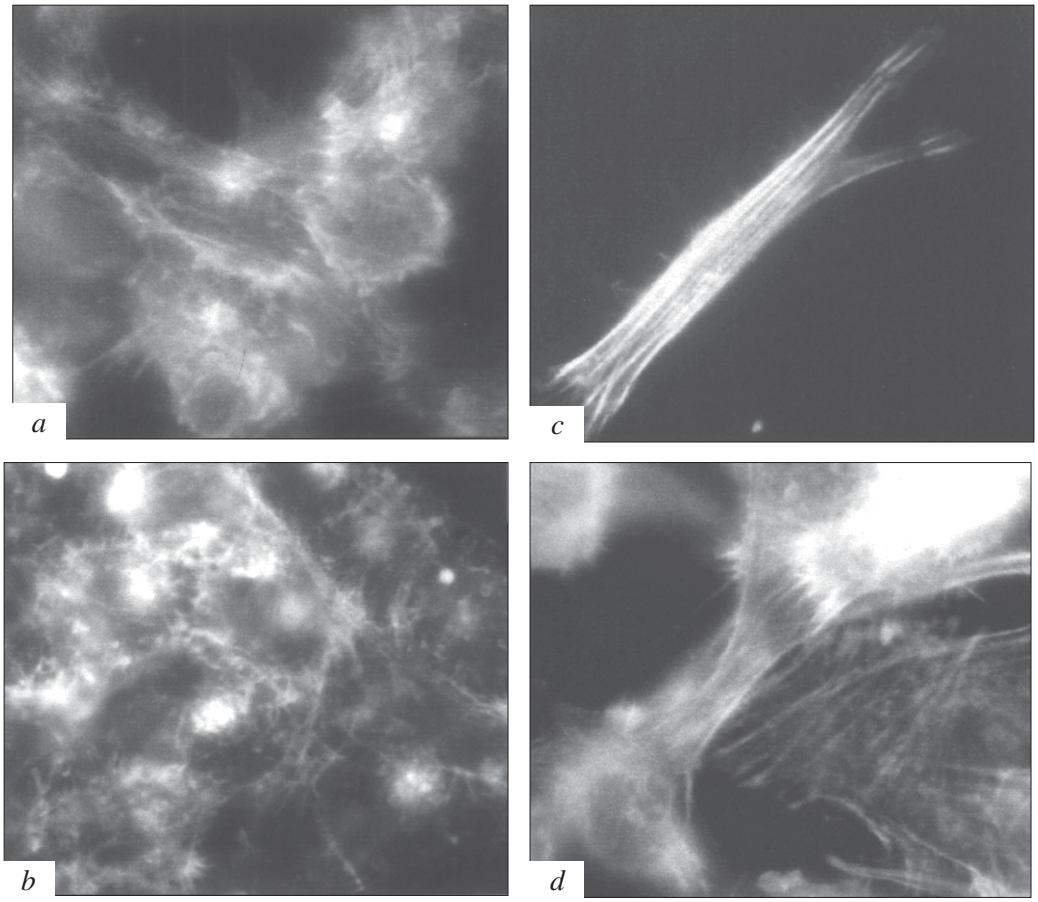

Fig. 1. Reorganization of actin microfilaments in 3T3-SV40 fibroblasts after treatment with N-acetylcysteine (NAC) and its removal, ×100. Fluorescent microphotographs of actin stained with phalloidin-TRITC. *a*) control; *b*) 24 h after addition of 10 mM NAC into culture medium; *c*, *d*) 24 h after removal of NAC; emergence of clear-cut stress fibrils is seen.

during and after NAC treatment. As we showed previously, NAC increased ROS content in the cells and produced a prooxidant effect within the first 4-5 h, which can explain disorganization of microfilaments [2]. Long-term (18-24 h) exposure of 3T3-SV40 cells to NAC reduces the intracellular level of ROS and increases GSH concentration (Table 1). However, removal of NAC from the culture medium did not crucially modified the situation for the next 20-24 h. The level of ROS somewhat increased, that of GSH decreased, but both parameters did not reach the control levels, which attested to persisting shifts in cell redox balance towards ROS decrease (Table 1). It is therefore difficult to explain, why stress fibrils emerge only after removal of NAC. It seems that the route of microfilament recovery was other than the route of their destruction.

Invasion of *E. coli* A2 in 3T3-SV40 cells persisted in the presence of NAC (Fig. 2). However, the bacteria do not penetrate into cells cultured for 24 h in medium after removal of NAC (Fig. 2, *c*). Hence, aftereffect of NAC manifesting in organization of actin into well-expressed stress fibrils correlates with the appearance of resistance to bacterial invasion, which, like stress fibrils, is typical of nontransformed cells [5]. Acquisition of morphological features and functional characteristics of normal cells by transformed cells suggests partial reversion of the transformed phenotype as a result of NAC aftereffect.

Partial morphological reversion of the transformed phenotype under the effect of some agents was known long ago and today is explained by changes either at the level of transcription or translation of this or that protein, including oncogenes, or at the level of proteasomal degradation [11]. It is clear that partial reversion caused, for example, by cAMP [9] or interferon indicates triggering of signal routes depending on these molecules. We can just say that the effect of NAC is not related to any known signal mechanism and can be regarded as a direct (recovery of cell surface protein SH groups) and indirect (through increased intracellular content of GSH and decrease in ROS content) influence. Both can modify activities of certain sig-

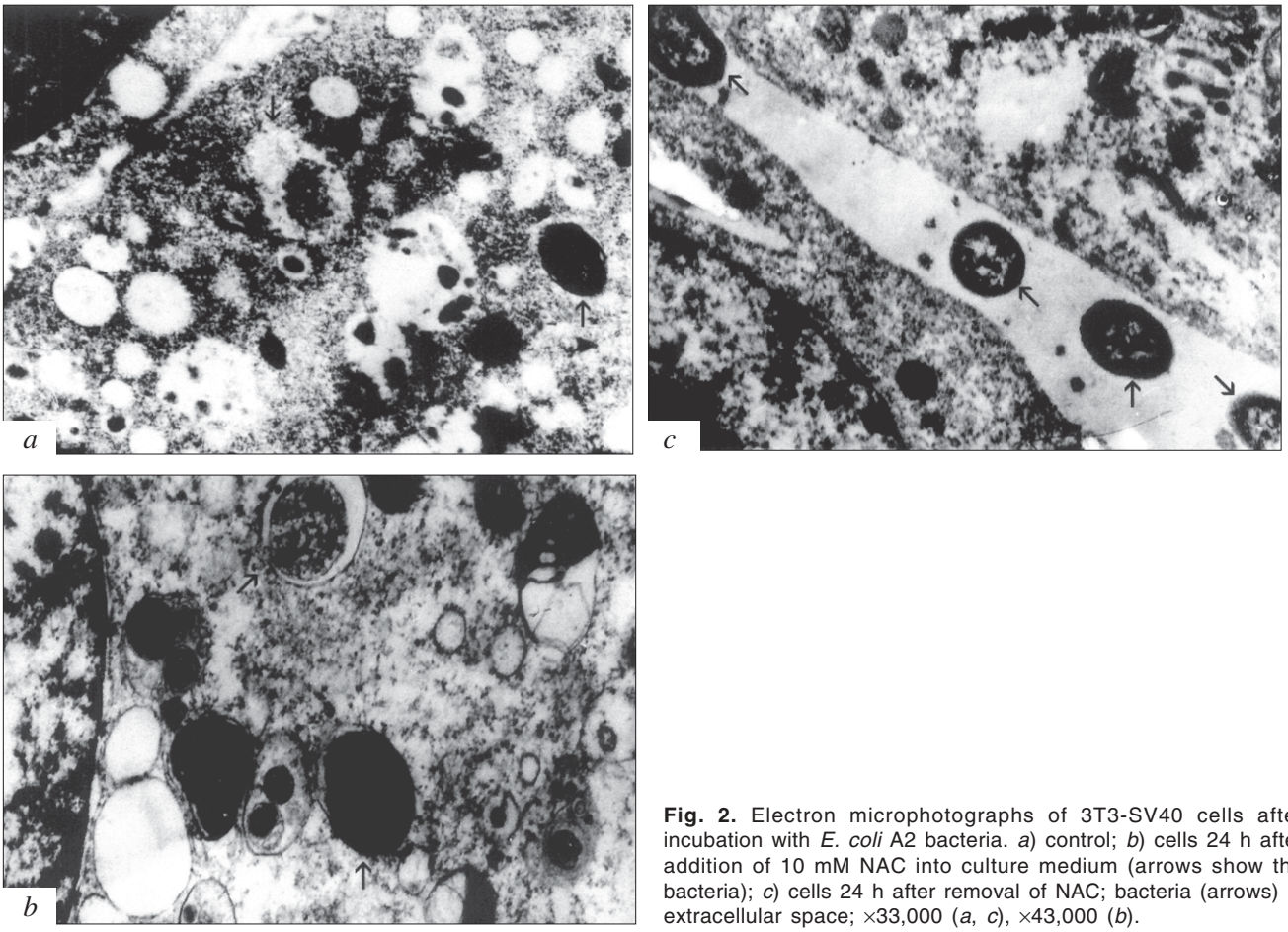

Fig. 2. Electron microphotographs of 3T3-SV40 cells after incubation with *E. coli* A2 bacteria. *a*) control; *b*) cells 24 h after addition of 10 mM NAC into culture medium (arrows show the bacteria); *c*) cells 24 h after removal of NAC; bacteria (arrows) in extracellular space; ×33,000 (*a*, *c*), ×43,000 (*b*).

nal molecules and involve modification of the pattern of synthesized proteins, eventually leading to reorganization of cell structures, specifically, of the cytoskeleton. The molecular mechanisms underlying the effect of NAC on cells, including transformed cells, can be different [15]. Our data indicating that long-term (24 h) exposure of 3T3-SV40 cells to NAC does not arrest their cell cycle (in contrast to the effect on nontransformed cells) [1] prove that transformed genotype of the cells is retained during exposure to this agent. Our further studies are aimed at elucidation of the NAC target, essential for restructuring of actin cytoskeleton and cell interactions with bacteria.

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REFERENCES

1. I. A. Gamalei, N. D. Aksyonov, T. N. Efremova, and K. M. Kirpichnikova, *Tsitologiya*, **45**, No. 1, 26-33 (2003).
2. T. N. Efremova, K. M. Kirpichnikova, S. Yu. Khaitlina, and I. A. Gamalei, *Ibid.*, **46**, No. 5, 395-403 (2004).
3. P. Cossart and P. J. Sansonetti, *Science*, **304**, No. 5668, 242-248 (2004).
4. P. N. Dekhuijzen, *Eur. Respir. J.*, **23**, No. 4, 629-636 (2004).
5. T. N. Efremova, N. Ender, M. Brudnaja, *et al.*, *Cell. Biol. Intern.*, **25**, No. 4, 557-561 (2001).
6. X. A. Gibson, A. Shartava, J. McIntyre, *et al.*, *Blood*, **91**, No. 11, 4373-4378 (1998).
7. M. Kirk and S. Pace, *Emerg. Med. Clin. North. Am.*, **15**, No. 2, 427-449 (1997).
8. L. Kum-Tatt and I. K. Tan, *Clin. Chim. Acta*, **53**, No. 2, 153-161 (1974).
9. J. F. Oram, A. J. Mendez, J. Lymp, *et al.*, *J. Lipid Res.*, **40**, No. 10, 1769-1781 (1999).
10. M. Tyunder, L. Susini, S. Prieur, *et al.*, *Proc. Natl. Acad. Sci. USA*, **99**, No. 23, 14,976-14,981 (2002).
11. L. Tylicki, B. Tutkowski, and W. H. Horl, *Kidney Blood Press. Res.*, **26**, Nos. 5-6, 303-314 (2003).
12. P. Velge, E. Bottreau, B. Kaeffer, and P. Pardon, *Med. Microbiol. Immunol.*, **183**, No. 3, 145-158 (1994).
13. P. Velge, E. Bottreau, N. Van-Langendonck, and B. Kaeffer, *J. Med. Microbiol.*, **46**, No. 8, 681-692 (1997).
14. I. T. Virlos, J. Mason, D. Schoefield, *et al.*, *Scand. J. Gastroenterol.*, **38**, No. 12, 1262-1267 (2003).
15. M. Zafarullah, W. Q. Li, J. Sylvester, and M. Ahmad, *Cell. Mol. Life Sci.*, **60**, No. 1, 6-20 (2003).