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N-ACETYLCYSTEINE AUGMENTS SURFACE THIOLS AND DIFFERENTIALLY MODULATES CELL ADHESION AND INVASION IN VITRO AND METASTATIC POTENTIAL IN VIVO OF B16F1 MELANOMA

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The redox state of surface protein thiols influences a variety of cell functions, and we recently reported that adhesion molecules can be redox regulated. We investigated the effect of reducing surface thiols using N-acetylcysteine (NAC) on the biological properties of murine melanoma B16F1 cells. Treating the cells with NAC (5mM for 2h, then removed by washing) augmented their capacity to adhere to fibronectin, as well as to adhere to and invade an endothelial cell monolayer. This was associated with an augmented expression of reduced surface protein thiols. However, when control or NAC-pretreated melanoma cells were injected i.v. in mice to induce experimental lung metastases, we could observe an inhibition of metastatic potential by NAC. This discrepancy suggest that other redox sensitive steps, in addition to adhesion, are important in regulating the metastatic phenotype in vivo.

Thiol antioxidants affect cell functions not only by scavenging free radicals but also through modification of the redox state of protein cysteines. In particular, we recently reported that NAC augments the expression of surface thiols of Jurkat cells in the reduced state, and augments adhesion to fibronectin, possibly by reducing the cysteines of integrins (1).

The thiol antioxidant and GSH repletor, NAC, has a variety of effects on cancer cells proliferation, apoptosis, tumorigenicity and metastatic potential, as well as on carcinogenesis (2) and inhibits formation of experimental metastasis in vivo in mice. (3-6).

Since adhesion is an early step in metastasis formation, and in consideration of the effect of NAC on surface thiols, we investigated its effect on adhesive and invasive properties of melanoma cells. For this purpose, we studied the effect of NAC treatment in vitro on the redox state of membrane protein thiols in B16F1 murine melanoma cells. In these experimental conditions, we studied the adhesion of melanoma cells to fibronectin or to murine endothelial cells. Finally, we studied the effect of in vitro exposure of melanoma cells to NAC in their ability to invade an endothelial cell monolayer and to induce experimental lung metastasis following i.v. injection in syngeneic mice.

In these studies we have used NAC as a tool to investigate the role of the redox state of the cell membrane in metastatic activity. In all experiments we removed by washing NAC from cancer cells to avoid any interference originating from an effect of NAC on the host. The results obtained indicate that the antimetastatic action of NAC is not mediated by an effect on the adhesive and invasive properties of the melanoma cells.

MATERIAL AND METHODS

Materials

NAC and fibronectin were from Sigma (St

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Louis, MO). NAC was prepared freshly before use and its solution was neutralized with NaOH before adding to the cells. N- (biotinoyl)-N- (iodoacetyl) ethylendiamine (BIAM) was from Molecular Probes (Eugene, OR). EZ-Link NHS-LC-Biotin ® was from Pierce Biotechnology (Rockford, IL, USA), streptavidin-peroxidase was from Roche Diagnostics GmbH (Mannheim, Germany), ABTS was from Euroclone (West York, UK), Percoll was from Amerham Pharmacia Biotech (Little Chalfont. UK).

Mice

Male C57BL/6N mice, 13 weeks of age, were obtained from Charles River, Calco, Italy. Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national (D.L. n. 116, G.U., suppl. 40, Feb. 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec.12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). The protocols for the proposed investigation were reviewed and approved by the Animal Care and Use Committees (IACUC) of the Istituto di Ricerche Farmacologiche "Mario Negri".

Cell lines

The B16F1 murine melanoma cell line was obtained from Dr. R. Giavazzi, Bergamo, Italy. Cells were maintained in DMEM containing 10% FCS. The murine endothelioma cell line sEnd.1 was obtained through the courtesy of Dr. E. F. Wagner (IMP, Wien, Austria) (7-8) and maintained and grown as described (9).

Determination of cell-surface protein thiols with BIAM-ELISA

Confluent B16F1 cells in 96-wells tissue culture plates (Becton Dickinson-Falcon, Franklin Lakes, NJ) were incubated with freshly prepared NAC in medium at a final concentration of 5mM, for 2h at 37°C. Then, the cells were washed twice with PBS, incubated with 100 μM BIAM in 100 μl of PBS for 15 min. The cells were then fixed with 50 μl of 3% formaldehyde in PBS for 15 min, and washed with PBS. Then, 100 ml/well of streptavidin-peroxidase, at a 1:10,000 dilution in PBS, were added and after 1 hour plates were read at 405 nm using ABTS as the peroxidase substrate according to the manufacturer's instructions. Data are expressed as absorbance at 405.

Cell adhesion

Cells were trypsinized, left for 30 min. at 37°C at 1x106/ml in complete medium to recover, and then treated with NAC as indicated above. After treatment, the cells were labeled with EZ-Link NHS-LC-Biotin® that forms a stable amide bond with primary amines at pH 7-9 (10). After washing, the cells were resuspended in medium at a concentration of 2.5 x 106/ml, and added (0.2 ml/well) to 96-wells plates coated with 50 μl of 4 mg/ml fibronectin. In some experiments, the same number of B16F1 cells were added to 96-well plates containing a monolayer of sEnd cells, prepared by plating sEnd cells (2x104 cells/0.2 ml/well) 24h before the experiment.

The plates were then incubated 30 min at 37°C, then non-adherent cells were eliminated by Percoll flotation and adherent cells fixed with glutaraldehyde as described by Goodwin and Pauli (11). Finally, the wells were washed three times with PBS, 100 ml/well of streptavidin-peroxidase, at a 1:10,000 dilution in PBS, were added and after 1 hour, plates were read at 405 nm using ABTS as the peroxidase substrate according to the manufacturer's instructions.

In vitro invasion of endothelial cell monolayer

Invasion of an endothelial cell monolayer by cancer cells was assayed according to published methods (12-13). Briefly, sEnd cells were grown in T75 flaskws until confluence, then culture medium was replaced with fresh medium. Then, B16F1 cells, pretreated with and without NAC for 2h as described, were overlayered on sEnd cells and cultured for 5 days. The invasion capacity of B16F1 cells was quantified by microscopically counting the penetrating colonies.

Experimental metastasis in mice

B16F1 cells (2 x 105 cells/200 μl/mouse) were

injected i.v. into the tail vein of C57BL/6N mice. To determine the number of metastatic nodules, the mice were sacrificed by cervical dislocation, the lungs were excised and fixed in Bouin's solution for 5 days. Tumour colonies on the surface of each of the 5 lobes were counted under a dissection microscope as described (14).

RESULTS

NAC augments reduced surface thiols, and adhesion of B16F1 cells to fibronectin and endothelial cells.

In the experiments shown in Fig. 1A, we studied the effect of NAC on surface thiols of B16 cells. Cells were treated with 5mM NAC for 2 h, then washed with PBS and surface thiols were measured using the BIAM-ELISA method. It can be seen that NAC shifted the redox state of surface proteins towards a reduced condition as the concentration of surface thiols augmented by 3-fold. In all these experiment, the viability of the cells, as judged by Trypan blue exclusion, was unaffected by NAC treatment and in several experiments was about 90%.

The effect of NAC on the adhesive properties of B16 cells was then investigated. For this purpose, cells were treated with NAC as described above, then washed to remove the chemical, and then allowed to adhere for 30 min to fibronectin-coated plastic. Then non-adherent cells are removed as described in Materials and Methods and adherent cells are quantified. As shown in Fig.1B, reductants augmented the adhesion to fibronectin by approximately two-fold. Using the same conditions, we studied the ability of these cells to adhere to a monolayer of endothelial cells. As shown in Fig 1C, also in this model, NAC augmented cell adhesion.

NAC treatment augments the in vitro invasion

Figure 2A shows representative pictures of B16 cells invading an endothelial cell monolayer. It can be seen that the number of penetrated colonies was higher with NAC-treated cells than with untreated cells. It is important to note that also in these experiment, cancer cells were treated with NAC and washed as described above to remove the chemical from the cells before adding them to the endothelial cell monolayer. A quantitative analysis of this effect, by counting different fields, is shown in Fig.2B.

NAC treatment diminishes in vivo metastasis

The ability of control- or NAC-treated melanoma cells to produce metastasis after iv injection in mice was then investigated. In these experiments, the cells were pre-treated in vitro with 5mM NAC for 2h, under the same experimental conditions used for the adhesion and invasion experiments described above, then NAC was removed by washing to avoid observing the effect of NAC on the host. The cells were then re-suspended in PBS and

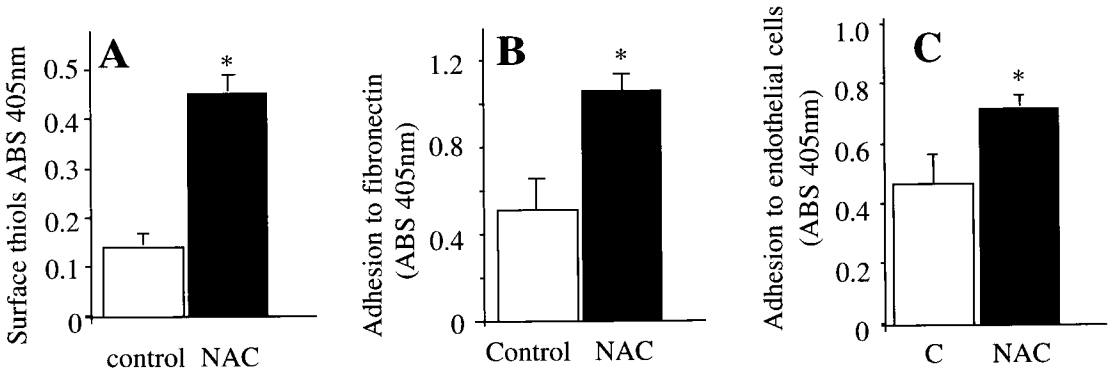

Fig. 1. *NAC treatment of melanoma cells augments their surface thiol expression (A), adhesion to fibronectin (B) and endothelial cells (C). Assays were preformed on cells 2 h after treatment with 5 mM NAC as described "material and methods". Data are mean ± S.E. (n=3), * P<0.01 Vs. control.*

injected iv. As shown in Fig. 3, NAC-treated cells produced significantly less lung metastasis, as evaluated 8 days later.

DISCUSSION

The most striking conclusion coming from these experiments is that NAC has opposite effects on a set of in vitro properties of B16F1 cells, namely adhesion and invasion, that are increased by NAC treatment, and their in vivo metastatic potential, which is decreased by NAC.

This different effect cannot be ascribed to differences in the treatment schedule as the cells were treated with NAC, which was then washed away, under exactly the same experimental conditions both for the in vitro and the in vivo experiments.

It is possible that this discrepancy reflects a different kinetics in these biological phenomena. In fact, cell adhesion, which is evaluated after one hour, and in vitro invasion, are rather direct consequences of the biological properties of the cells. It is likely that since adhesion is the first essential step in the invasion process (15), regulating adhesion will also result in changes in the in vitro invasion. This would explain how an increase in the redox state of surface thiols, that others and we have shown to directly influence the adhesive properties of the cell, increases their invasiveness.

On the other hand, in vivo metastatization, that is evaluated several days after injection, is likely the resultants of several factors with different kinetics. In fact, the first step in the formation of metastasis is the arrest of the cells in the small capillaries of the target organ, followed by adhesion, extravasation, and proliferation reviewed in references (16-18), and, obviously, survival. Some of these steps, particularly entrapment, occur in the very first minutes after injection of the cells, while others, namely proliferation, take several days. Furthermore, several factors associated with the host response, including immunity and inflammation, intervene to regulate the process.

NAC could affect many of these steps. For instance, cell deformability is a key determinant for the trapping of the cells in the microvasculature (18-19). Reactive nitrogen species were suggested to increase sequestration of cancer cells in the lung by decreasing their deformability (19), and several works have shown that a pro-oxidant status is associated with an increased viscosity and rigidity of the cell membrane (20-21). Furthermore, in vitro treatment of cancer cells with NAC was shown to increase their immunogenicity (4). The observation that NAC directly inhibit gelatinase activity (3) might also represent a further effect that explains our observations.

In conclusion, the results obtained in vitro suggest that the redox state of surface protein thiols is a key determinant in the adhesive properties of the tumour cells and, consequently, in their ability to invade an endothelial cell monolayer. However, in

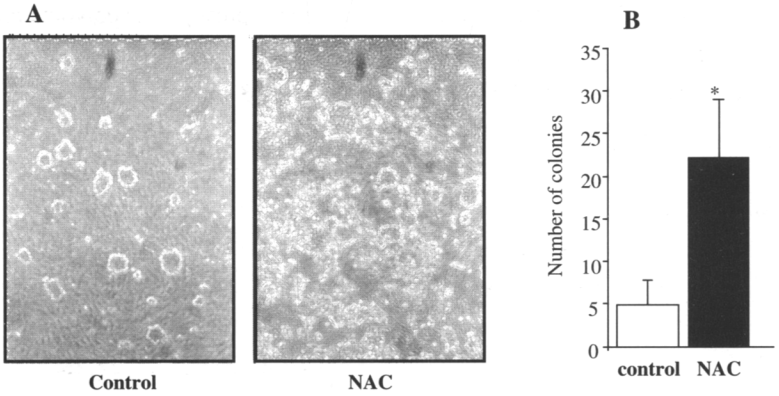

Fig. 2. *NAC increases in vitro invasion of sEnd cells by melanoma cells. A: photomicrographs of melanoma cells invading sEnd monolayers. B: quantitation of the in vitro invasion. Melanoma cells were treated with 5mM NAC for 2h. The results are expressed as average number of penetrating colonies in nine fields ± SD. *P<0.05 Vs. control.*

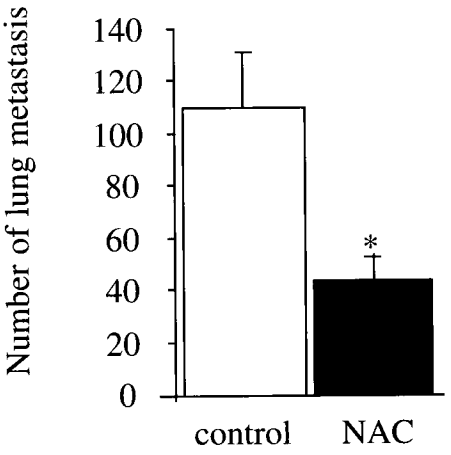

Fig. 3. *Experimental metastasis in mice injected i.v. with control- or NAC-treated melanoma cells. Cells were treated with 5 mM NAC for 2h, then washed, resuspended in PBS and 2 x 105 cells were injected iv in mice. Lung colonies were counted 8 days later. Data are mean ± S.D. (control, n=10; NAC, n=6); P< 0.005 Vs. control.*

the specific case of NAC, the results reported here indicate that, when studying the effect of thiol antioxidants on metastatic ability of cancer cells, in vitro tests of adhesivity and invasiveness are not predictive of the effects in vivo. It is important to investigate further which are the steps in metastasis formation that are regulated by NAC to identify those redox regulated protein thiols of the cells that are important in the spread of cancer.

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REFERENCES

1. **Laragione T., V. Bonetto, F. Casoni, T. Massignan, G. Bianchi, E. Gianazza and P. Ghezzi.** 2003. Redox regulation of surface protein thiols: identification of integrin alpha-4 as a molecular target by using redox proteomics. *Proc. Natl. Acad. Sci. USA 100:14737.*
2. **De Flora S., A. Izzotti, F. D'Agostini and R. M. Balansky**. 2001. Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points. *Carcinogenesis 22:999.*
3. **Albini A., F. D'Agostini, D. Giunciuglio, I. Paglieri, R. Balansky and S. De Flora.** 1995. Inhibition of invasion, gelatinase activity, tumor take and metastasis of malignant cells by N-acetylcysteine. *Int. J. Cancer 61:121.*
4. **Goldman Y., A. Peled and M. Shinitzky.** 2000. Effective elimination of lung metastases induced by tumor cells treated with hydrostatic pressure and N-acetyl-L-cysteine. *Cancer Res. 60:350.*
5. **Futakuchi M., K. Ogawa, S. Tamano, S. Takahashi and T. Shirai.** 2004. Suppression of metastasis by nuclear factor kappaB inhibitors in an in vivo lung metastasis model of chemically induced hepatocellular carcinoma. *Cancer Sci. 95:18.*
6. **De Flora S., F. D'Agostini, L. Masiello, D. Giunciuglio and A. Albini.** 1996. Synergism between N-acetylcysteine and doxorubicin in the prevention of tumorigenicity and metastasis in murine models. *Int. J. Cancer 67:842.*
7. **Williams R. L., W. Risau, H. G. Zerwes, H. Drexler, A. Aguzzi and E. F. Wagner.** 1989. Endothelioma cells expressing the polyoma middle T oncogene induce hemangiomas by host cell recruitment. *Cell 57:1053.*
8. **Williams R. L., S. A. Courtneidge and E. F. Wagner.** 1988. Embryonic lethalities and endothelial tumors in chimeric mice expressing polyoma virus middle T oncogene. *Cell 52:121.*
9. **Bussolino F., M. De Rossi, A. Sica, F. Colotta, J. M. Wang, E. Bocchietto, I. M. Padura, A. Bosia, E. DeJana and A. Mantovani.** 1991. Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines. *J. Immunol. 147:2122.*
10. **Butler J. E., J. E. Spradling, M. Suter, S. E. Dierks,**

H. Heyermann and J. H. Peterman. 1986. The immunochemistry of sandwich ELISAs I. The binding characteristics of immunoglobulins to monoclonal and polyclonal capture antibodies adsorbed on plastic and their detection by symmetrical and asymmetrical antibody-enzyme conjugates. *Mol. Immunol. 23:971.*

11. **Goodwin, A. E., and B. U. Pauli.** 1995. A new adhesion assay using buoyancy to remove non-adherent cells. *J. Immunol. Meth. 187:213.*
12. **Ohigashi H., K. Shinkai, M. Mukai, O. Ishikawa, S. Imaoka, T. Iwanaga and H. Akedo.** 1989. In vitro invasion of endothelial cell monolayer by rat ascites hepatoma cells. *Jpn. J. Cancer Res. 80:818.*
13. **Asensi M., I. Medina, A. Ortega, J. Carretero, M. C. Bano, E. Obrador and J. M. Estrela.** 2002. Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Radic. Biol. Med. 33:387.*
14. **Pisano C., G. Pratesi, D. Laccabue, F. Zunino, P. Lo Giudice, A. Bellucci, L. Pacifici, B. Camerini, L. Vesci, M. Castorina, S. Cicuzza, G. Tredici, P. Marmiroli, G. Nicolini, S. Galbiati, M. Calvani, P. Carminati and G. Cavaletti.** 2003. Paclitaxel and Cisplatin-induced neurotoxicity: a protective role of acetyl-L-carnitine. *Clin. Cancer Res. 9:5756.*
15. **Okahara H., H. Yagita, K. Miyake and K. Okumura.** 1994. Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor alpha enhancement of experimental metastasis. *Cancer Res. 54:3233.*
16. **Hood J. D. and D. A. Cheresh.** 2002. Role of integrins in cell invasion and migration. *Nat. Rev. Cancer 2:91.*
17. **Liotta L. A. and E. C. Kohn.** 2001. The microenvironment of the tumour-host interface. *Nature 411:375.*
18. **Mundy G. R.** 2002. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer 2:584.*
19. **Igawa S., I. Hayashi, N. Tanaka, H. Hiruma, M. Majima, T. Kawakami, M. Hirose, N. Masuda and H. Kobayashi.** 2004. Nitric oxide generated by iNOS reduces deformability of Lewis lung carcinoma cells. *Cancer Sci. 95:342.*
20. **Hong J. H., M. J. Kim, M. R. Park, O. G. Kwag, I. S. Lee, B. H. Byun, S. C. Lee, K. B. Lee and S. J. Rhee.** 2004. Effects of vitamin E on oxidative stress and membrane fluidity in brain of streptozotocin-induced diabetic rats. *Clin. Chim. Acta 340:107.*
21. **Choe M., C. Jackson and B. P. Yu.** 1995. Lipid peroxidation contributes to age-related membrane rigidity. *Free Radic.Biol.Med. 18:977.*