Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 83-88

IDENTIFICATION OF NATURAL KILLER ENHANCING FACTOR AS A MAJOR ANTIOXIDANT IN HUMAN RED BLOOD CELLS

Hungyi Shau[1] and Anthony Kim

Division of Surgical Oncology and Jonsson
Comprehensive Cancer Center, UCLA Medical Center
Los Angeles, CA 90024-1782

Received December 14, 1993

SUMMARY: Human natural killer enhancing factor genes (NKEF-A and B) share extensive homology with several genes from other organisms known to be induced by oxidative stress and to protect organisms and proteins from oxidative destruction. In this study, we have identified natural killer enhancing factor as an abundant protein in red blood cell cytosol. Highly purified natural killer enhancing factor blocks inactivation of enzymes by mixed metal-thiol oxidation. Thus, natural killer enhancing factor is a major antioxidant protecting red blood cells from oxidative injuries.

We previously identified a human protein, natural killer enhancing factor (NKEF), from red blood cells (RBCs) which enhances NK cytotoxicity against tumor cells. We recently cloned NKEF genes from a human erythroleukemic cell line K562 and found that NKEF genes (A and B) share extensive homology with each other (75% identical at the amino acid level)[2,3]. In addition, both NKEF-A and B share extensive identity with a yeast protein termed thiol-specific antioxidant (TSA) (2). TSA protects yeast from oxidative insults (3). An in vitro assay system for TSA takes advantage of

[1]To whom correspondence should be addressed: Hungyi Shau, Ph.D., 54-140 CHS, Division of Surgical Oncology, UCLA Medical Center, Los Angeles, CA 90024-1782. FAX: 310-206-3610.

[2]GenBank accession numbers are L19184 for NKEF-A and L19185 for NKEF-B.

[3]Shau, H., Butterfield, L. H., Chiu, R. and Kim, A. Cloning and sequence analysis of candidate human natural killer enhancing factor genes. manuscript submitted.

Abbreviations used: ahp-c, C22 subunit of alkyl hydroperoxide reductase; NKEF, natural killer enhancing factor; RBCs, red blood cells; ROI, reactive oxygen intermediates.

0006-291X/94 $5.00

its ability to protect glutamine synthetase and other enzymes from inactivation by reactive oxygen intermediates (ROI) and/or thiol radicals generated in the mixed metal-thiol oxidation system (3). It is believed that ROI and thiol radicals are generated in the system by the following reactions: [1] $R\text{-}SH + Fe^{3+} \text{-----} > R\text{-}S^{\cdot} + H^{+} + Fe^{2+}$ [2] $Fe^{2+} + O_2 \text{-----} > Fe^{3+} + O_2^{-\cdot}$ [3] $2\ O_2^{-\cdot} + 2\ H^{+} \text{-----} > H_2O_2 + O_2$ [4] $R\text{-}S^{\cdot} + R\text{-}SH \text{-----} > R\text{-}S\text{-}S\text{-}R^{-\cdot} + H^{+}$ [5] $R\text{-}S\text{-}S\text{-}R^{-\cdot} + H_2O_2 \text{----} > R\text{-}S\text{-}S\text{-}R + OH^{-} + OH^{\cdot}$ This chain reaction can be blocked by cation chelators like EDTA or by hydrogen peroxide scavengers like catalase. Indeed, both EDTA and catalase protect glutamine synthetase from inactivation in the system. Unlike catalase, TSA does not directly scavenge hydrogen peroxide (3). Therefore, TSA represents a new class of natural antioxidants in protecting cells from oxidative insults. Because of the significant shared identity between TSA and NKEF, we investigated the antioxidant activity of NKEF in this study.

Materials and Methods

Preparation of RBCs, cytosol and NKEF. RBCs from normal donors and RBC cytosol were prepared as previously described (1). NKEF was highly purified by ammonium sulfate precipitation and Q-Sepharose Fast Flow column as before (1). All protein samples were dialyzed in 50 μM of imidazole HCl buffer (pH 7.0) before testing.

Depletion of NKEF and SDS-PAGE analysis. Rabbit anti-human NKEF antibodies were generated as previously described (1). One ml of the antiserum was bound to 5 ml of Pharmacia (Alameda, CA) protein G Sepharose 4 Fast Flow beads to create a NKEF-specific antibody column. Four mg of RBC cytosol proteins in 1.5 ml of 20 mM sodium phosphate buffer (pH 7.0) were loaded onto the anti-NKEF antibody column, and the proteins not bound to the antibodies were washed off the column with the same buffer. SDS-PAGE analysis of proteins was performed under reducing condition and the gels were stained by Coomassie Blue as reported (1).

Antioxidant activity of NKEF and mixed thiol-metal oxidation of glutamine synthetase. Antioxidant activity of NKEF was measured by its ability to protect glutamine synthetase from inactivation by mixed metal-thiol oxidation. Glutamine synthetase function was indicated by its glutamyltransferase activity (2,3), performed in microwells. NKEF protein at indicated concentration was mixed with glutamine synthetase (1.5 units/ml), 33 μM of $FeCl_3$, and 27 mM of 2-mercaptoethanol as thiol reagent in a total volume of 150 μl of 50 mM imidazole HCl buffer (pH 7.0). After 15 min incubation at 37°C, 50 μl of glutamine synthetase substrate containing 150 mM glutamine, 400 μM ADP, 20 mM potassium arsonate, 20 mM of neutralized NH_2OH HCl, and 400 μM $MnCl_2$ in imidazole buffer was added. Following 30 more mins of incubation, 50 μl of stop solution containing 55 g/l of $FeCl_3$, 20 g/l of trichloric acid, and 105 ml/l of HCl was added. The plate was read with an ELISA plate reader at 550 nm wave length.

Reagents. Unless indicated otherwise, the chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Result

NKEF is an abundant protein in RBC. In SDS-PAGE analysis, we observed that the protein with an apparent molecular mass of 22-24 kDa, same as that of NKEF, was the third or fourth most abundant protein band in the whole RBC cytosol (Fig. 1). To further identify this abundant protein, we pass RBC cytosol through the antibody column specific for human NKEF. The 22-24 kDa band is mostly depleted by the anti-NKEF column, further indicating that this abundant protein in RBC cytosol is NKEF (Fig. 1).

Antioxidant function of NKEF. Both NKEF-A and B share extensive homology with the yeast TSA. Using the Bestfit program of the University of Wisconsin's Genetics Computer Group software, we found that TSA shares 57% identity and 70% similarity with NKEF-A, and 66% identity and 75% similarity with NKEF-B at the amino acid level (data not shown). It is not yet known whether the native NKEF from RBC cytosol is the product of NKEF-A or B, or a mixture of both.

Since NKEF and the yeast TSA share extensive homology with each other, we tested for the antioxidant activity of NKEF. Treatment with the mixed metal-thiol oxidative system inactivates most of the glutamyltransferase activity of glutamine synthetase

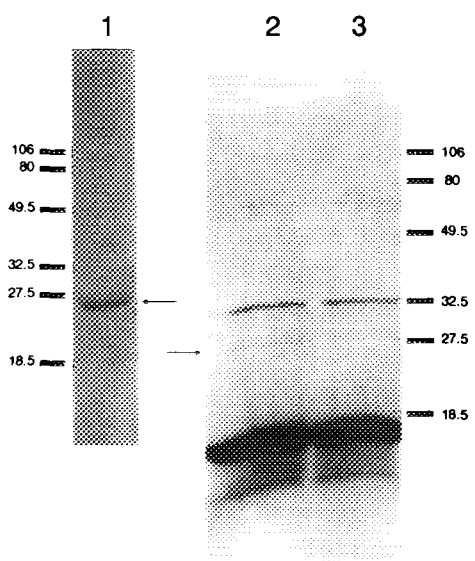

Fig. 1. SDS-PAGE analysis of highly purified NKEF (lane 1), RBC cytosol (lane 2), and RBC cytosol passed through anti-NKEF antibody column (lane 3). The amount of protein loaded was 7 µg for lane 1, and 20 µg for lane 2 and 3. The arrows indicate the position of 22-24 kDa NKEF band.

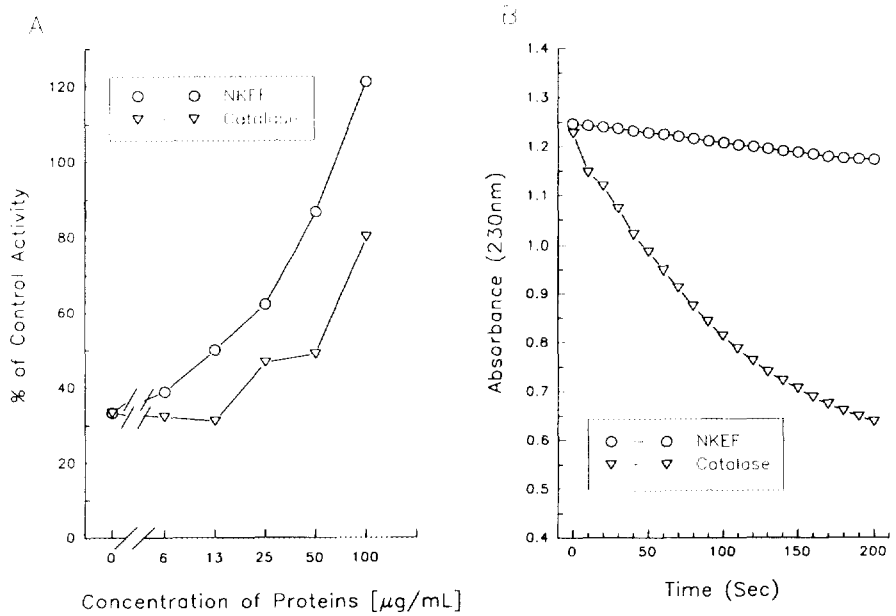

Fig. 2. (A) NKEF and catalase protect glutamine synthetase from inactivation by mixed metal-thiol oxidation. (B) Testing for catalase-like activity of NKEF. NKEF or control bovine catalase (15 μg/ml) was mixed with 0.06% H_2O_2. The catalase activity is measured by decrease of A_{230} every 10 sec.

(Fig. 2A) (3). However, in the presence of NKEF, glutamine synthetase is protected from the oxidative inactivation. The protective activity of NKEF is dose-dependent. Higher concentrations of NKEF provide greater protection of glutamine synthetase from oxidative damage. Similar dose dependent antioxidant function of NKEF was observed in five separate experiments. NKEF itself has no glutamine synthetase activity (data not shown). Like NKEF, the hydrogen peroxide scavenger catalase also protects glutamine synthetase from inactivation by mixed metal-thiol oxidation. However, on the basis of unit protein weight, NKEF is at least twice as effective as catalase in protecting glutamine synthetase.

We also tested for the catalase-like activity of NKEF. Results in Fig. 2B show that NKEF cannot directly scavenge hydrogen peroxide. In contrast, catalase at the same concentration rapidly eliminates hydrogen peroxide. These results were repeated in three other similar experiments. Therefore, NKEF protects enzymes from mixed metal-thiol oxidative inactivation via a mechanism distinct from direct elimination of hydrogen peroxide in the system.

Discussion

Comparison with available sequences in the GenBank data base shows that NKEF genes share extensive homology with several other genes from a variety of organisms at both the nucleotide and amino acid levels[2,3]. Besides the yeast TSA, these NKEF-related proteins include the MER5 (4) and MSP23 proteins (5) from mouse, a 29-kDa protein from Entamoeba histolytica (6), a 26-kDa protein from Helicobacter pylori (7), a product coded by an open reading frame in the genome of Clostridium pasterurianum (GenBank accession #M60116) and the 22-kDa subunit of alkyl hydroperoxide reductase (ahp-c) from Salmonella typhimurium and Escherichia coli (8,9). The percent identity of amino acid sequences between NKEF and the related proteins from other species ranges from the low of 30% between ahp-c and NKEF-B to the high of 93% between MSP23 and NKEF-A. Prosperi et al recently cloned the cDNA for a human proliferation-associated gene (pag) (10) which shares 97% identity with NKEF-A at the amino acid level and may be identical to NKEF-A.

The biological function of NKEF-related proteins is largely unknown. Prosperi et al (10) suggested that pag is important for cell growth because it is overexpressed in proliferating cells. Inhibition of MER5 expression suppresses the differentiation of mouse erythroleukemic cells (11). The fact that these genes are so well conserved and that their protein products are so abundant in some of the species where they are identified (6,7) indicates that they serve a very fundamental and essential function for the survival of cells throughout evolution.

The most striking common feature of many of the NKEF-related genes is that oxidative stress induces their hyperexpression (3,5,8,9,12-14). Furthermore, TSA and ahp are responsible for the survival of the yeast and the bacteria, respectively, in high concentrations of alkyl hydroperoxides. The data that NKEF and TSA directly protect enzymes from oxidative inactivation confirm that these gene products are natural antioxidants. The fact that these gene products share no homology with known scavengers of ROI and do not directly scavenge ROI indicates that they are a new class of natural antioxidants.

Acknowledgments

We thank Dr. Sidney Golub for his advice, discussion and support. This work was supported by grants from the TRDRP of the

University of California, Stein/Oppenheimer Foundation and Forman Foundation.

References

1. Shau, H., Gupta, R.K., and Golub, S.H. (1993) Cell. Immunol. 147, 1-11.
2. Chae, H.Z., Kim, I-H, Kim, K, and Rhee, S.G. (1993) J. Biol. Chem. 268, 16815-16821.
3. Kim, K., Kim, H. H., Lee, K-Y, Rhee, S.G., and Stadtman, E. R. (1988) J. Biol. Chem. 263:4704-4711.
4. Yamamoto, T., Matsui Y., Natori S., and Obinata, M. (1989) Gene 80, 337-343.
5. Ishii, T., Yamada, M., Sato, H., Matsue, M., Taketani, S., Nakayama, K., Sugita, Y., and Bannai, S. (1993) J. Biol Chem. 268, 18633-18636.
6. Torian, B.E., Flores, B.M., Stroeher, V.L., Hagen, F.S., and Stamm, W.E. (1990) Proc. Natl. Acad. Sci. USA 87, 6358-6362.
7. O'Toole, P.W., Logan S.M., Kostrzynska, M., Wadstrom, T., and Trust, T.J. (1991) J. Bacteriol. 173, 505-513.
8. Taraglia, L.A., Storz, G., Brodsky, M.H., Lai, A., and Ames, B.N. (1990) J. Biol. Chem. 265, 10535-10540.
9. Storz, G., Jacobson, F.S., Tartaglia, L.A., Morgan, R.W., Silveira, L.A., and Ames, B.N. (1989) 171, 2049-2055.
10. Prosperi, M-T., Ferbus, D., Karczinski, I., and Goubin, G. (1993) J. Biochem. Biol. 268, 11050-11056.
11. Nemoto Y., Yamamoto T., Takada S., Matsui Y., and Obinata, M. (1990) Gene 91, 261-265.
12. Kim, I. H., Kim, K., and Rhee, S. G. (1989) Proc. Natl. Acad. Sci. USA 86, 6018-6022.
13. Sato, H., Ishii, T., Sugita, Y., Taeishi, N., and Bannai, S. (1993) Biochim. et Biophysic. Acta 1148, 127-132.
14. Yamaguchi, M., Sato, H., and Bannai, S. Biochem. Biophysic. Res. Commun. 193, 1198-1201.