Enzyme Release from Cultured Human Melanoma Cells

E. KARG,[1]* B. HULTBERG,[2] A. ISAKSSON,[2] E. ROSENGREN[3] and H. RORSMAN[1]

Departments of [1]Dermatology, [2]Clinical Chemistry and [3]Pharmacology, University of Lund, Sweden (*on leave from the Department of Dermatology, Univ. Med. Sch. Pécs, Hungary)

The lysosomal enzyme β-hexosaminidase and the melanocyte specific enzyme tyrosinase were examined in human melanoma cell cultures. The β-hexosaminidase activity of the medium was approximately 40% of the total cellular activity after 24 h, while after 48 h the activity in the medium was twice that of the cells. The tyrosinase activity in the medium was 5% and 19% of the total cellular activity after the 24 h and 48 h incubation, respectively. The low level of lactate dehydrogenase activity in the medium after 24 as well as 48 h of incubation indicated that the release of β-hexosaminidase and tyrosinase was not due to membrane injury. The data suggest, that 1) β-hexosaminidase may be a candidate for tumor markers in malignant melanoma, and 2) the tyrosinase activity found in sera of melanoma patients may be due, at least partly, to enzyme release by living cancer cells. *Key words: β-hexosaminidase; Lysosomal enzyme; Tyrosinase; Tumor markers.*

Biochemical and immunochemical tumor markers are of potential importance in the diagnosis and management of cancers. An increasing number of human tumors has been shown to contain elevated levels of the lysosomal enzyme, β-hexosaminidase (EC. 3.2.1.30) (1–3). Studies of β-hexosaminidase have proved the existence in most tissues of two major isoenzymes which are referred to as the A and B forms for their respective acidic and basic properties (4). Both isoenzymes have N-acetyl-β-glucosaminidase and N-acetyl-β-galactosaminidase activity. In human colonic carcinomas the B isozyme was found to be increased in relation to the A isozyme (3) and a functionally atypical, variant form was demonstrated in metastatic tumor tissue of human liver (5).

Besides the higher tissue levels, elevated activity of β-hexosaminidase was reported in the gastric juice in the case of gastric carcinoma (6) and in the urine of patients with lung carcinoma (7). Furthermore, studies of cancer patients with different primary sites and metastatic involvement suggested that serum β-hexosaminidase may be a useful marker for monitoring the progression of malignant disease (8). However, there is no specific information about β-hexosaminidase in correlation with malignant melanoma.

On the other hand, the role of tyrosinase (EC. 1.14.18.1.) as a marker of malignant melanoma is relatively well established (9–11). This enzyme catalyses the initial steps in melanin synthesis and it has its origin exclusively in melanocytes. Tyrosinase activity was reported by Sohn et al. (9) in serum of a patient with melanosis due to disseminated melanoma. Nishioka et al. (10) found tyrosinase in sera from melanoma patients but not in sera from patients with other malignancies. Agrup et al. (11) have shown detectable quantities of tyrosinase in five out of seven sera from patients with widespread melanoma metastasis, but not in patients with other types of cancer.

The source of tyrosinase and elevated β-hexosaminidase activities in the sera may be the necrotizing tumor tissue, but the elevation may also be due to release from living cancer cells. In the present study tyrosinase and β-hexosaminidase releases were examined in the culture of a cell line of malignant melanoma (IGR 1). Lactate dehydrogenase (EC. 1.1.1.27.) was assayed in order to detect enzyme leakage due to membrane defect.

MATERIALS AND METHODS

Culture

Cultures of a pigment producing melanoma cell line (IGR 1) were obtained from Dr. Christian Aubert, Marseilles, and have been kept since March, 1982 in culture at the Tornblad Institute, University of Lund using methods previously described (12). The medium used was Eagle's minimal essential medium (MEM) with 15% fetal calf serum. About 0.75×10^6 cells were seeded in 5 ml medium in
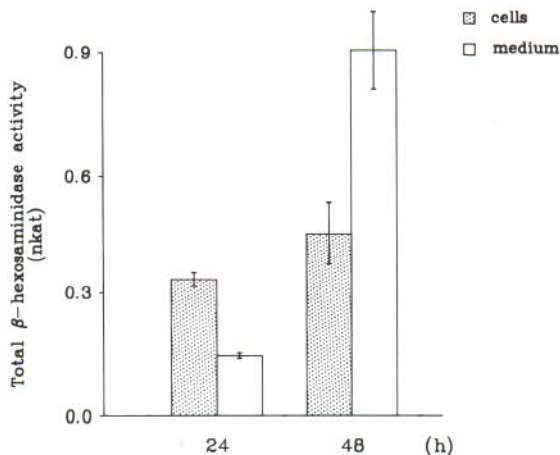
Fig. 1. Time-related changes of the total β-hexosaminidase activity in cultured melanoma cells and in the medium. □: cells, □: medium. (Mean ± S.D. of 5 samples).

Fig. 2. Time-related changes of the total tyrosinase activity in cultured melanoma cells and in the medium. □: cells, □: medium. (Mean ± S.D. of 10 samples).

polystyrene culture flasks, volume 50 ml. Cells were allowed to attach and grow for 24 and 48 h, respectively. The doubling time was approximately 24 h.

Enzyme assays

Before harvesting, the media were removed and the flasks were washed twice with a phosphate buffered saline solution, pH 7.2. For the β-hexosaminidase and lactate dehydrogenase assays cells were collected in the same buffer using a rubber policeman. After centrifugation (180 × g, 10 min), the pellets were stored at −20°C until analysis, when they were homogenized in 1 ml 10 mM sodium phosphate buffer, pH 6.0, containing 100 mM sodium chloride in an all-glass Potter Elvehjem homogenizer.

The media removed from the cells were centrifuged (180 × g, 10 min) and the supernatants stored in the same way as the cell samples. Enzyme activities were also determined in culture media incubated for the same time period without cells. The difference of the values obtained from the media incubated with and without cells was considered to be the enzyme activity released from cells.

Determinations of β-hexosaminidase and its isoenzymes were earlier described (13). Activity of lactate dehydrogenase was assayed in accordance with the methods of the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (14).

Tyrosinase was extracted from the cells by adding 3 ml 0.2% Triton-X in 0.5 M phosphate buffer, pH 7.4 to the flasks. After incubation for 30 min at room temperature the samples were homogenized and frozen to −20°C. Determinations of the enzyme activity (15) were performed in aliquots quantifying the amount of 5-S-L-cysteinyl-L-dopa specifically produced when 2 mM D, L-dopa was incubated with 3 mM L-cysteine in 0.5 M phosphate buffer, pH 7.4, maintained at 37°C and gently aereated. The contents of 5-S-L-cysteinyl-L-dopa were determined by HPLC with electrochemical detection. This compound represents about 80% of the cysteine adducts of dopaquinone besides
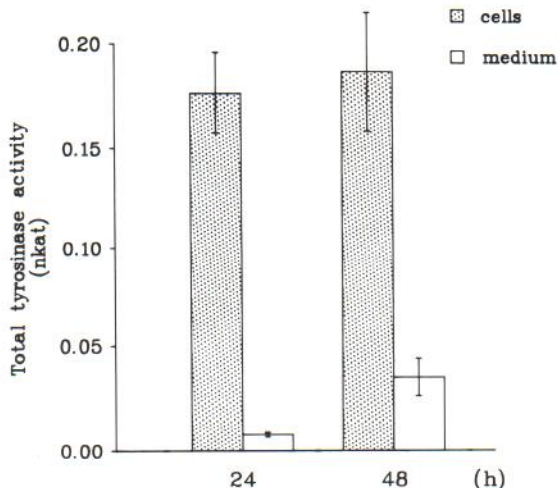
the 2- and 6-S-cysteinyl isomers. Tyrosinase activity was calculated by multiplying the quantity of 5-S-L-cysteinyl-L-dopa by a factor of 1.25.

The enzyme activities were related to total cell content per flask, to total medium per flask and to mg cell protein, respectively.

Protein was analysed according to Lowry et al. (16).

RESULTS

The total cellular β-hexosaminidase activity and the enzyme activities in the medium are shown in Fig. 1.
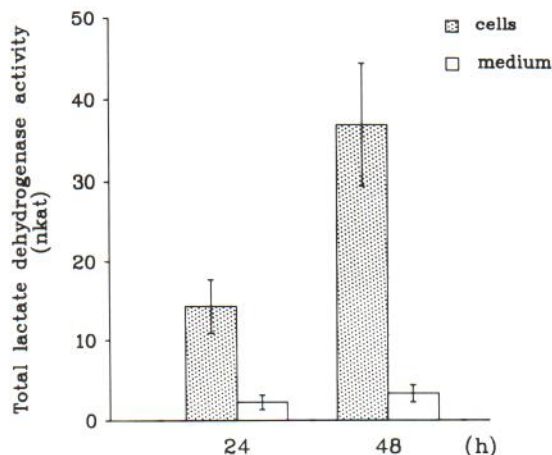

Fig. 3. Time-related changes of the total lactate dehydrogenase activity in cultured melanoma cells and in the medium. □: cells, □: medium. (Mean ± S.D. of 5 samples).

Table. I. *Changes in the protein related activities of β-hexosaminidase, tyrosinase and lactate dehydrogenase in cultured human melanoma cells.* Values are expressed as nkat per mg cell protein. (Mean ± S.D. of a=5 and b=10 samples).

Time (h)	β-Hexosaminidase[a]	Tyrosinase[b]	Lactate dehydrogenase[a]
24	1.1±0.05	0.36±0.03	47±11
48	0.8±0.14	0.23±0.03	64±13

The medium/cell ratio of the enzyme activity was 0.42 (24 h) and 1.98 (48 h). According to the isoenzyme determinations the cellular B isozyme activities at 24 h and 48 h were 15.6 ± 1.5% and 17.8 ± 2.6% of the total enzyme activity. In the medium the isozyme values were not measurable at 24 h, but at 48 h the B isozyme was 29.0 ± 4.1%.

Since the percentage of B isozyme in the media relative to the cells was found to be increased, we tested the stability of the two isozymes. Conditioned media removed after 24 and 48 h of culture were incubated without cells at 37°C for 4 h. Under these conditions there was a 60–80% loss of the A isozyme activity whereas the B isozyme was unaffected.

The total cellular tyrosinase activity and the corresponding values in the medium are shown in Fig. 2. The medium/cell ratio of the enzyme activity was 0.05 (24 h) and 0.21 (48 h), respectively.

The total cellular lactate dehydrogenase activity and the enzyme activity in the medium are shown in Fig. 3. The medium/cell ratio of the enzyme activity was 0.15 (24 h) and 0.09 (48 h).

The enzyme activity of β-hexosaminidase related to protein weight was 1.1 ± 0.05 nkat/mg cell protein (24 h) and 0.8 ± 0.14 nkat/mg cell protein (48 h), (mean ± S.D of n=5 samples) (Table I.). The corresponding values of tyrosinase activity were 0.36 ± 0.03 nkat/mg cell protein (24 h) and 0.23 ± 0.03 nkat/mg cell protein (48 h), (mean ± S.D of n=10 samples). The lactate dehydrogenase activity related to protein weight was 47 ± 11 nkat/mg cell protein after 24 h incubaton and 64 ± 13 nkat/mg protein after 48 h of incubation (mean ± S.D. of n=5 samples).

DISCUSSION

The most striking observation from our study was the high level of β-hexosaminidase activity in the medium after 48 h in contrast to the melanoma cells. The β-hexosaminidase activity found in the medium after 24 h of culture corresponded to about 40% of the total cellular activity, while after 48 h the activity in the medium was twice that of the cells. This very high release was not due to cell membrane injuries, since the lactate dehydrogenase release in 48 h of culture was only about 9% of the intracellular activity. The pronounced elevation of β-hexosaminidase activity in the culture medium is likely to be due to a preferential release of newly synthetized enzymes.

The increase of B isozyme in the medium relative to the cells probably depends on the fact that the A isozyme is more labile as indicated by the loss of its activity in the conditioned medium. Furthermore, the lability of the A isozyme indicates that the release of β-hexosaminidase from the melanoma cells is in fact higher than the measurable enzyme activity in the medium.

All lysosomal enzymes examined to date are initially synthetized in the rough endoplasmic reticulum as precursor polypeptides that are transported to the Golgi complex (17). The mannose-6-phosphate (Man-6-P) recognition marker present on many lysosomal hydrolases is responsible for selective and efficient targeting of these proteins to the lysosomal compartment (18). The marker is added to oligosaccharides on the precursor in the endoplasmic reticulum and Golgi apparatus by a two-step process that involves transfer of N-acetyl-glucosamine-1-phosphate to mannose residues and conversion of the resulting phosphodiester moieties to monoester forms (Man-6-P) by removal of the terminal N-acetyl-glucosamine residue. As the phosphorylated enzymes move through the Golgi apparatus, they bind to Man-6-P receptors. This specific ligand-receptor interaction allows sorting of lysosomal hydrolases from other glycoproteins and specific transport of the enzymes to lysosomes where they are subsequently dephosphorylated and proteolytically processed to generate lysosomally localized mature enzymes (17, 18).

Generally a small fraction of the newly synthetized precursors of lysosomal enzymes becomes secreted, usually 5–20% (17). But in some cell types up to 50% of the precursor forms can be secreted to the medium. A comparison of the secreted activity

with the total intracellular enzyme activity reveals that most cell types including fibroblasts secrete about 5% of the total cellular enzyme per day (17).

Relatively massive secretion of lysosomal enzymes (predominantly mature forms) occurs in macrophages in cell cultures after activation. Thioglycollate – elicited macrophages secrete lysosomal enzyme corresponding to 25% of the total cellular enzyme per day (17). Recent studies have shown that also under certain other conditions, lysosomal enzymes can be secreted in large quantities and that the secreted enzymes may function extracellularly in such processes as iron transport, bone resorption, cell growth and tumor metastasis (19–22).

The invasion of normal tissue by malignant cells is usually characterized by the destruction of normal tissue components in the vicinity of the invading tumor cells. It is well established that lysosomal enzymes are important for breakdown of protein and glycoconjugates and there is growing evidence that lysosomal enzymes may play a significant role in tumor invasion (21–22).

Carr (23) has demonstrated that administration of aldonolactones, inhibitors of β-glucuronidase and β-N-acetyl-glucosaminidase, caues regression of tumors (S. 37, Ehrlich tumor and tumor 2146) in mice. He suggested that aldonolactones, by their effects on glycosidases, inhibit penetration of intracellular cement substance by the tumor cells. In accordance with this Sinowatz et al. (24) suggested that β-hexosaminidase activity was greater in more anaplastic, invasive carcinomas. However, Whitehurst et al. (25) have found lower activity in poorly differentiated prostatic tumors.

Whatever the function of lysosomal glycosidases in tumor progression, cultured melanoma cells seem to release a very high amount of β-hexosaminidase in culture. It would be of interest to examine the sera of melanoma patients with regard to β-hexosaminidase activity and metastatic involvement, since this enzyme may prove to be a useful tumor marker.

The tyrosinase activity of the medium in relation to the intracellular activity was much lower than that of β-hexosaminidase. However, the increasing medium/cell ratios in comparison with the corresponding lactate dehydrogenase values show that the melanma cells release tyrosinase into the medium. The data also suggest that the tyrosinase activity found in the sera of melanoma patients may be due, at least partly, to enzyme release by living tumor cells.

The release of tyrosinase, just like that of β-hexosaminidase, may be correlated with the tumor properties of the melanoma cells. But it is possible that such release of enzymes also occurs in normal melanocytes.

ACKNOWLEDGEMENTS

REFERENCES

1. Conchie J, Lewy GA. Comparison of different glycosidase activities in conditions of cancer. Br J Cancer 1957; 11: 487–493.
2. Bosmann HB, Hall TC. Enzyme activity in invasive tumors of human breast and colon. Proc Natl Acad Sci USA 1974; 71: 1833–1837.
3. Brattain MG, Pretlow TP, Pretlow TG II. Cell fractionation of large bowel cancer. Cancer 1977; 40: 2479–2486.
4. Robinson D, Sterling JL. N-acetyl-β-glucosaminidases in human spleen. Biochem J 1968; 107: 321–327.
5. Alhadeff JA, Holzinger RT. Presence of an atypical thermolabile species of β-hexosaminidase B in metastatic-tumour tissue of human liver. Biochem J 1982; 201: 95–99.
6. Graffner H, Hultberg B. Carcinoembryogenic antigen and lysosomal enzymes in gastric juice as an aid in the diagnosis of gastric cancer. J Surg Oncol 1983; 24: 233–235.
7. Brattain MG, Kimball PM, Durant JR, Pretlow TG II, Smith D, Carpenter J, Marsk M. Urinary hexosaminidase in patients with lung carcinoma. Cancer 1979; 44: 2267–2272.
8. Plucinsky M, Porok JJ, Alhadeff JA. Variant serum β-hexosaminidase as a biochemical marker of malignancy. Cancer 1986; 58: 1484–1487.
9. Sohn N, Gang H, Gumport SL, Goldstein M, Deppisch LM. Generalized melanosis secondary to malignant melanoma. Report of a case with serum and tissue tyrosinase studies. Cancer 1969; 24: 897–903.
10. Nishioka K, Romsdahl MM, McMurtrey MJ. Adaptation of triatiated tyrosinase assay to serum tyrosinase and its specific elevation in melanoma. In: Klaus SN, ed. Pigment Cell, vol. 5. Basal: Karger, 1979: 300–304.
11. Agrup P, Carstam R, Wittbjer A, Rorsman H, Rosengren E. Tyrosinase activity in serum from patients with malignant melanoma. Acta Derm Venereol (Stockh) 1989; 69: 120–124.
12. Aubert C, Lagrange C, Rorsman H, Rosengren E. Catechols in primary and metastatic human malignant melanoma cells in monolayer culture. Eur J Cancer 1976; 12: 441–445.

13. Isaksson A, Hultberg B, Masson P, Löw K, Sundgren E. Lundblad A. Enzyme immunoassay of β-hexosaminidase isoenzymes using monoclonal antibodies. Scand J Clin Lab Invest (in press).

14. The Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology. Recommended methods for the determination of four enzymes in blood. Scand J Clin Lab Invest 1974; 33: 291–312.

15. Wittbjer A, Dahlbäck B, Odh G, Rosengren A-M, Rosengren E, Rorsman H. Isolation of human tyrosinase from cultured melanoma cells. Acta Derm Venereol (Stockh) 1989; 69: 125–131.

16. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951; 193: 265–275.

17. Skudlarek MD, Novak EK, Swank RT. Processing of lysosomal enzymes in macrophages and kidney. In: Dingle IT, Dean RT, Sly W, eds. Lysosomes in biology and pathology, vol. 7. Amsterdam: Elsevier, 1984: 17–44.

18. Creek KE, Sly WS. The role of phospho-mannosyl receptor in the transport of acid hydrolases to lysosomes. In: Dingle IT, Dean RT, Sly WS, eds. Lysosomes in biology and pathology, vol. 7. Amsterdam: Elsevier, 1984: 63–82.

19. Baumbach GA, Saunders PTK, Bazer FW, Roberts M. Uteroferrin has N-asparagine-linked high-mannose-type oligosaccharides that contain mannose-6-phosphate. Proc Natl Acad Sci USA 1984; 81: 2985–2989.

20. Vaes G. On the mechanisms of bone resorption. J Cell Biol 1968; 39: 676–697.

21. Poule AR. Tumor lysosomal enzymes and invasive growth. In: Dingle JT, ed. Lysosomes in biology and pathology, vol. 3. Amsterdam: Elsevier, 1973: 303–337.

22. Liotta L, Thorgeirsson UP, Garbisa S. Role of collagenases in tumor cell invasion. Cancer Metastasis Rev 1982; 1: 277–288.

23. Carr AJ. Effect of some glycosidase inhibitors on experimental tumours in mouse. Nature 1963; 198: 1104–1105.

24. Sinowatz F, Weber P, Gasser G, Mossig H, Skolek-Winnisch R. A histochemical study of glycosidases in benign prostatic hyperplasia and in prostatic carcinoma in the human. Urol Res 1978; 6: 103–107.

25. Whitehurst GB, Mashburn JP, Pretlow TG II, Bradley EL, Boohaker EA. Prostatic hexosaminidase activity in patients with benign prostatic hyperplasia and prostatic carcinoma. Cancer Res 1982; 42: 4300–4303.