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TRANSGLUTAMINASE ACTIVITY DURING THE DIFFERENTIATION OF MACROPHAGES[1]

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SUMMARY Transglutaminase (R-glutaminyl-peptide:amine γ-glutamyltransferase, E.C. 2,3,2,13) activity during the differentiation of murine leukemia cell lines (Ml cells) was investigated. Ml cells contained a significant transglutaminase activity of the tissue type. During the course of differentiation into mature macrophage-like Ml^+ cells induced with a protein inducer, the enzymatic activity was stimulated more than ten times as much as in the original undifferentiated Ml^- cells. A remarkable enhancement of enzymatic activity was also observed when lipopolysaccharide was utilized as an inducer of differentiation. The enzymatic activity of undifferentiated Ml^- cells was eluted at the region of M.W. ca. 80,000 as a single and symmetrical peak on Sepharose 4B column chromatography. By contrast, most of the activity in differentiated Ml^+ cells was eluted at the void volume under the same condition, though some activities were eluted at the same region as in Ml^- cells. These data suggest that most of transglutaminase activity exists in the form of a high-molecular-weight complex with some cellular components in differentiated cells. The possible physiological significance of the enzyme in macrophage functions was discussed.

INTRODUCTION

Many of the physiological functions of macrophages involve cell membranes, such as phagocytosis, tumoricidal activity, and 'helping' activity towards lymphocytes in antibody production. Thus, it is of interest to study the change in membrane properties during the differentiation and functional maturation of macrophages. Ml cells have been used as a model for the study of macrophage differentiation (1-4). This cell line was first established from spontaneous myelogenous leukemia cells in SL/Am mice, and can differentiate into mature

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Abbreviations used: EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol.

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macrophage-like cells in vitro when cultured with various inducers (5). We and other investigators have recently reported that Ml cells acquire most of the important functions of normal mature macrophages upon differentiation (2-4), and invetigated some of the biochemical events underlying the differentiation process (1, 6-8).

Transglutaminase (R-glutaminyl-peptide:amine γ-glutamyltransferase, E.C. 2,3,2,13) catalyzes the crosslinking of proteins through γ-glutamyl ε-lysine bridges (9-11), reportedly plays a significant role in membrane protein cross-linking, and is suggested to affect the physical property of biomembranes (12-14). It has also been reported that this enzyme is involved in the process of receptor-mediated endocytosis (15,16), which is, as phagocytosis, one of the most important functions of mature macrophages. The existence of this enzyme in immunocompetent cells has already been reported (17). In this paper, in order to clarify some of the biochemical events underlying the differentiation of macrophages, we have explored the transglutaminase activity in Ml cells during the course of differentiation.

MATERIALS AND METHODS

Cells Ml$^-$ cells were maintained as myeloblastoid cells (doubling time ca. 20 hours) in Eagle's minimum essential medium with a double concentration of amino acids and vitamins (Nissui Seiyaku Co., Tokyo) supplemented with 10 % inactivated horse serum in a humidified atmosphere of 10 % CO_2. Differentiated type Ml$^+$ cells, having high activities in phagocytosis and locomotion, were obtained by the 48 hour culture of Ml$^-$ cells with a protein inducer prepared from the culture supernatant of the secondary culture of murine embryo fibroblasts as described previously (1-5). In some experiments, lipopolysaccharide (final 5 μg/ml) was used as an inducer of differentiation.

Assay of transglutaminase activity. Transglutaminase activity was assayed by measuring the amount of [1,4-^{14}C]putrescine incorporated into a protein acceptor (18,19). Cells were washed three times with phosphate buffered saline (pH 7.4) containing 1 mM EDTA and 2 mM DTT. Cells were sonicated in this suspension with a Branson Sonifier (Branson Sonic Power, Plainview, N.Y.) at output setting at 5 for 5 min. The protein concentration was determined according to the method of Lowry et al. (20). The standard assay mixture (0.5 ml) contained 50 mM Tris-HCl buffer (pH 8.5), 1 mM EDTA, 5 mM $CaCl_2$, 10 mM DTT, 0.02 % casein (Casein nach Hammersten, Merck, FRG), and 10 μM [1,4-^{14}C]putrescine (0.5 μCi). The reaction was started by the addition of 0.1 to 0.4 mg protein. Incubation was carried out at 37 °C for 60 min unless otherwise stated. Reaction was stopped by the addition of trichloroacetic acid (final 5 %) and cold putrescine (final 2 mM). Precipitates formed were collected on a filter paper and washed twice with 5 % trichloroacetic acid followed with absolute ethanol and acetone. The radioactivity of samples was measured using an Aloka scintillation counter (Aloka, Kobe, Japan). The specific activity of transglutaminase was calculated by subtracting the background radioactivity (usually less than 10 % of the experimental values) from the experimental value. Background activity was determined either by using an enzyme preparation heated at 100 °C for 5 min, or through the deletion of $CaCl_2$ from the incubation mixture.

Chemicals. [1,4-^{14}C]putrescine (specific radioactivity, 102-122 mCi/mmol) was obtained from Radiochemical Center (Amarsham, U.K.). Monodansylcadaverine was purchased from Sigma (St. Louis, MO). Putrescine, DTT and EDTA were obtained from Nakarai Chemicals (Kyoto, Japan). Lipopolysaccharide (E. coli 0127:B6) was purchased from DIFCO (Detroit, MI). Other chemicals were of reagent grade and the best quality commercially available.

RESULTS

Transglutaminase activities of Ml cells. The enzymatic activity was optimum at pH 8.5 to 9.0, absolutely Ca^{2+}-dependent and required more than 0.25 mM $CaCl_2$ for a maximal activity. The activity was linear up to 2 hours of incubation under the standard assay condition employed in this study. SH reagents such as iodoacetamide, N-ethylmaleimide or p-chloromercuribenzoic acid were in general the effective inhibitors (data not shown), and various amines also inhibited the incorporation of [^{14}C]putrescine into casein. The concentrations of amines which gave 50 % inhibition of putrescine incorporation in our assay system were, dansylcadaverine (74 μM), hydroxylamine (0.37 mM), methylamine (1.6 mM), ethylamine (3.8 mM) and isopropylamine (53 mM). These inhibitor profiles are generally in good agreement with those of the well-characterized tissue transglutaminases so far reported (9).

Transglutaminase activity during the differentiation of Ml cells. As shown in Fig.1, the transglutaminase activity was markedly enhanced by the differentiation of Ml cells. The differentiated type of cells (Ml^+ cells) usually contained more than ten times as much enzyme activity as in undifferentiated Ml^- cells. The protein inducer preparation used in these studies contained no significant transglutaminase activity. As shown in Fig. 1a, the addition of protein inducer to undifferentiated Ml^- cells produced a significant increase in enzymatic activity as early as 12 hours after the addition, and the activity linearly increased for 4 days. As reported previously (1,5), the cells acquired Fc-receptor (65 to 74 % of cells) and phagocytic activity (76 to 90 % of cells) 24 hours after the addition of protein inducer, and proliferative activity was significantly suppressed. The addition of lipopolysaccharide, known as an another inducer of Ml cell differentiation (3,4), produced a moderate increase in transglutaminase activity (Fig. 1b). After 24 hours of addition of lipopolysaccharide, 40 % of cells gained Fc-receptors and 65 % of cells acquired phagocytic activity. When

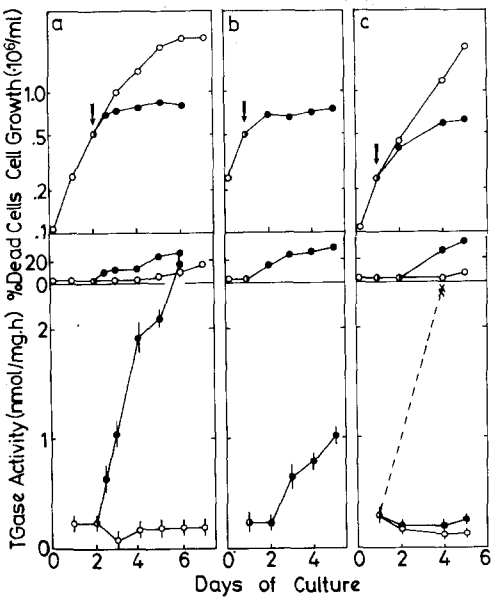

Fig. 1. Effect of a protein inducer (a), lipopolysaccharide (b) and low serum concentration (c) on the Ml cell transglutaminase activity. In (a), protein inducer was added to the culture on day 2 of culture (arrow); ●, differentiated Ml^+ cells; o, control Ml^- cells without protein inducer. In (b), lipopolysaccharide (final 5 μg/ml) was added to the culture on day 1 (arrow). In (c), the concentration of horse serum in culture medium was decreased to 0.5 % on day 1 of culture (arrow); ●, Ml^- cells cultured with 0.5 % serum; o, Ml^- cells with 10 % serum; x, differentiated Ml^+ cells cultured with 10 % serum and protein inducer under the same condition, serving as a positive control.

the concentration of horse serum in culture medium was decreased to 0.5 % in order to suppress cell proliferation, the arrest of the proliferative activity was apparent after 2 days, but the cells acquired no markers of differentiation. In this case the change in enzymatic activity was far less significant in comparison with those observed during differentiation (Fig. 1c).

Sepharose 4B column chromatography of Ml cell transglutaminase. When the sonicate of Ml^- cells was subjected to Sepharose 4B column chromatography, the enzymatic activity was eluted as a single and symmetrical peak at the region of M.W. ca. 80,000, showing the existence of tissue type transglutaminase (Fig. 2a). When the sonicate of Ml^+ cells induced with protein inducer was subjected to the column chromatography under the same condition, significant activity was eluted at the same region as with Ml^- cell sonicate, but most of the activity was eluted at the void volume (Fig. 2b). This phenomenon was observed equally in four independent experiments. No significant change was observed in the elution

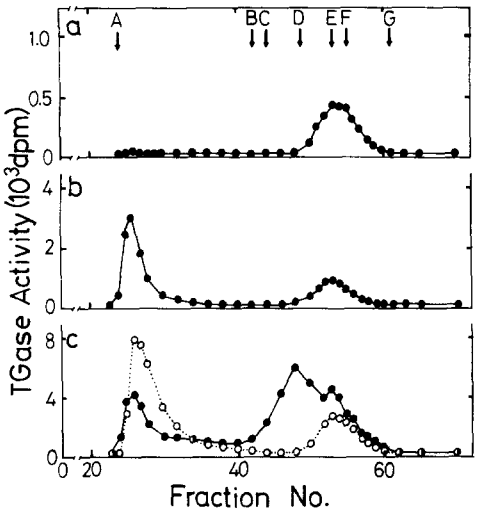

Fig. 2. Sepharose 4B column chromatography of Ml cell transglutaminase. 15 mg protein of each enzyme preparation was applied. Eluted with 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl and 1 mM EDTA. Column size; 1.4 x 50 cm (bed volume 76 ml); reference protein, A, blue dextran (M.W. >2000,000); B, ferritin (M.W. 450,000); C, bovine plasma transglutaminase (M.W. 320,000); D, aldolase (M.W. 158,000); E, guinea pig liver transglutaminase (M.W. 80,000); F, bovine serum albumin (M.W. 69,000); G, cytochrome C (M.W. 12,500). (a), Ml^- cell sonicate; (b), Ml^+ sonicate. In (c), Ml^+ cell sonicate was solubilized with Triton X-100 (final 0.5 %) and chromatographed in the presence of 0.1 % Triton X-100; ●—●, Ml^+ cell sonicate in the presence of detergent; o···o, without detergent.

pattern when the enzyme preparation from Ml^+ cells were chromatographed in the presence of 1 M KCl (data not shown). In the presence of a detergent, however, the enzymatic activity in void volume was significantly reduced to ca. 40 % of the original activity, and a new peak of activity appeared around the M.W. of 140,000 to 230,000 (Fig. 2c). Triton X-100 was the most effective detergent among tested, although the concentration of Triton X-100 more than 0.5 % was inhibitory to the enzymatic activity.

DISCUSSION

We describe in this paper an alteration in transglutaminase activity during the course of the differentiation of murine leukemia cells into mature macrophage-like cells. Transglutaminases occur in mammalian tissues as several types; the plasma type having the M.W. of 320,000 and tissue types with M.W. around 80,000 or less (9-11). Transglutaminase activity in Ml cells appeared as tissue type, judging from its M.W. and other characteristics. A marked increase in trans-

glutaminase activity was detected during the course of differentiation of Ml cells induced by protein inducer, and a similar enhancement of activity was observed when lipopolysaccharide was used as an inducer instead. Thus, it can be concluded that significant increase of transglutaminase activity is associated with Ml cell differentiation. Time course of an increase of enzymatic activity is in line with the increased induction of the enzyme during the course of differentiation.

Birckbichler et al. (21) reported the dependency of transglutaminase activity on the proliferative state of cells in culture. Since Ml cells lose their ability to proliferate upon differentiation, a similar mechanism could also be at work. However, when the growth of Ml cells was suppressed by decreasing the amount of horse serum in the culture medium, the increase in transglutaminase activity was far less significant. Thus, it appears that in Ml cells, changes in the transglutaminase activity are more intimately associated with differentiation of cells rather than the proliferative state of cells. We have recently observed that thioglycolate-stimulated murine peritoneal macrophages have 3 to 5 times as much transglutaminase activity as in non-stimulated resident peritoneal macrophages (Teshigawara, Noro, Kannagi and Masuda, unpublished). Thus an increase in transglutaminase activity seems to be a general phenomenon accompanying the differentiation and functional maturation of macrophages.

Interestingly, most of the transglutaminase activity was revealed to exist in the form of some high-molecular weight complexes in mature cells. These results indicate that some changes have occurred as to the natural state of the enzyme within cells during the course of differentiation, besides the simple increase in the enzymatic activity. It has been suggested that upon activation, transglutaminase itself can eventually serve as the substrate for its own enzymatic activity (22). If so, the enzyme would be covalently bound with each other or with other cellular substrate proteins when it works. Thus, the presence of the enzyme in the form of macromolecular complexes may be an evidence showing that the enzyme is much more actively at work in differentiated cells. It has already been reported that considerable enzymatic activity is associated with macromolecular structures in some cultured cell lines (23), but the biological significance of

this phenomenon has not been clarified. Our present data indicated that the enzyme is normally soluble and cytoplasmic in undifferentiated cells, and, during the course of differentiation into mature cell type, the enzyme increases in activity and becomes attached to some subcellular structures. These macromolecular structures were partially solubilized by the column chromatography in the presence of a detergent, and the results suggested that the protein or protein complex having the M.W. of 140,000 to 230,000 could be their unit structure. Or, these results could be also explained assuming another type of intracellular transglutaminase having a distinct M.W. from that of well known liver enzyme is present, as has been suggested by Chung et al. (unpublished, cited in reference 11). Further investigation is needed to elucidate this point.

It has been suggested that transglutaminase is involved in the process of receptor-mediated endocytosis (15,16). In agreement with this proposal, a high enzymatic activity and presence of high-molecular-weight complex were specifically observed in differentiated type cells, which have a high phagocytic activity. Another physiological function of the enzyme has been suggested to be the modification of cell-surface protein, especially cross-linking of membrane proteins (12-14). During the course of differentiation of macrophages, profound changes in membrane properties are known to occur; e.g., increased activity of Fc-receptors, or appearance of Ia antigens. It is also well known that these cell surface receptors and antigens play an important role in the 'helping' activity of macrophages towards T- and B-lymphocytes in producing specific antibodies, possibly through handling antigens at the cell surface. We have already shown that differentiated Ml^+ cells possess this helping activity, but undifferentiated Ml^- cells do not have this activity (2,3). In this context, it is of interest to note that some proteins in histocompatibility antigen complex can serve as substrate for the transglutaminase (24). Thus, transglutaminase may be relevant to those biological events at the surface of mature macrophage membranes.

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