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HIGH CONCENTRATION OF NEUTRAL ENDOPEPTIDASE (ENKEPHALINASE E.C. 3.4.24.11) IN A MALIGNANT TUMOR: RAT HEPATOMA 3924A

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The activity of the membrane-bound neutral endopeptidase 24.11 was low in the normal liver (21 ± 3 pmol/h/mg protein, mean ± SE) but it increased 56-fold in rapidly-growing rat hepatoma 3924A. The identity of the enzyme in the tumor was established by immunoprecipitation and by using a specific inhibitor of neutral endopeptidase. The endopeptidase concentration in the differentiating and regenerating liver was lower than in normal tissue, 39 and 8% of the corresponding control. The activity of a plasma membrane marker enzyme carboxypeptidase M in the normal liver was 1.0 ± 0.2 nmol/h/mg protein, it increased about 2-fold in the rapidly-growing hepatoma and in the differentiating liver, but was unchanged in regenerating liver. The function of the strikingly increased neutral endopeptidase activity in the rapidly growing hepatoma may relate to activation of autocrine or exocellular growth factors or to inactivation of cell proliferation-inhibitory factors. Such a biochemical change should confer selective advantages to the cancer cells.

Neutral endopeptidase (E.C. 3.4.24.11; NEP) is a metallopeptidase that cleaves peptides at the amino side of hydrophobic amino acids (1). It is widely distributed in the body. The microvillous structures in the brush border of kidney (2), intestine (3), placenta (4) or choroid plexus (5) are especially rich in this enzyme. NEP cleaves a wide range of bio-active peptides. Because it inactivates the opioid peptide enkephalins, its presence in the CNS and its inhibition there have been the subject of many studies (6,7). It was thought that NEP cleaves peptides of a molecular mass of less than 3 kDa, but recently the hydrolysis of 17 kDa (8) interleukin 1 by NEP was

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The abbreviations used are: NEP, neutral endopeptidase (EC 3.4.24.11); CPM, carboxypeptidase M; CALLA, common acute lymphoblastic leukemia antigen.

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reported. Research on NEP recently branched out in a different direction, after the discovery of the identity of CALLA with NEP (9-12). Thus NEP is present in lymphoblasts, but absent from mature lymphocytes. In contrast, in neutrophils, it occurs only in mature cells where it can cleave chemotactic peptides (13,14). To find out more about the presence and putative function of NEP in other types of malignant cells, we studied its activity in a solid tumor. We discovered that NEP activity which was very low in normal, differentiating and regenerating rat liver increased 56.6-fold in a rapidly growing hepatoma.

MATERIALS AND METHODS

Control, Normal Tissues. Tissues from a total of 29 rats were used in this study. The enzymic phenotype of the hepatomas was compared primarily with that of the normal liver of ACI/N (n=8) rats of the same sex, strain, age, weight, and nutritional status as the tumor-bearing ones (15). Livers from normal Wistar rats were also used in order to determine strain differences in enzymic activity (n=3). Animals were kept in individual cages with Purina fox chow and water available ad libitum and were killed between 9 and 11 a.m. Rats were under regulated cycles of light and darkness (15).

Regenerating Liver. It was essential to clarify whether the biochemical pattern was due to neoplastic alteration and progression or whether it reflected rates of proliferation that occur in normal liver (15). The biochemical phenotype of the proliferating normal liver was elucidated by studying the regenerating livers in adult ACI/N strain rats 24 h after partial hepatectomy (n=3). The livers of sham-operated normal ACI/N rats were used as controls (n=4). The proliferation rate of the regenerating liver was similar to that of hepatoma 3942A (15).

Differentiating Liver. This model in Wistar strain rats also provides a control for rapid proliferation rate in the postnatal developing rat (n=3). It was noted that livers should not be used before 6th day after birth because in the rat the fetal liver is largely a hemopoietic organ (16).

Hepatoma 3924A. This hepatoma was induced originally in ACI/N rats by feeding N-fluoro-4-biphenylacetamide (n=8) (17). The growth properties and biochemical phenotype of this malignant transplantable solid tumor have been reported (15,18).

Homogenization and Fractionation. Liver samples were weighed, minced and homogenized in 20 mM Tris, pH 7.4 and 0.25 M sucrose in a Waring blender. Homogenates were fractionated at 4°C by sequential centrifugation at 1,000 x g for 10 min, 10,000 x g for 25 min and 100,000 x g for 60 min. Pellets were washed by resuspension in the Tris/sucrose buffer followed by recentrifugation. The final washed pellets (designated P_1, P_2 and P_3, respectively) were resuspended in Tris/sucrose buffer, aliquoted and either assayed immediately or stored at -70 °C.

Enzyme Assays. NEP activity was measured as the phosphoramidon-inhibitable endopeptidase activity using glutaryl-Ala-Ala-Phe-methoxynaphthylamide as substrate at pH 6.5 in a two-step assay (13). Membrane-bound carboxypeptidase M (CPM) activity was measured as the 2-mercaptomethyl-3-guanidinoethylthio-

propanoic acid inhibitable activity using Dansyl-Ala-Arg as substrate at pH 7.5 in a fluorometric assay (19).

Protein Assay. The protein concentrations in cell fractions were determined by a routine method (20) with crystalline bovine serum albumin as standard.

Immunoprecipitation. Immunoprecipitation was carried out using Triton X-100 solubilized material employing rabbit anti-human NEP antiserum and Protein A as reported (21). Normal rabbit serum served as a control.

RESULTS AND DISCUSSION

Activity of NEP in the P_3 Fraction of Normal and Neoplastic Livers. Upon fractionation of the various liver samples, the NEP specific activity was highest in the P_3 tissue fractions where it was enriched 2.5-3.5 times over that of the whole homogenate. We therefore compared activity in the P_3 fractions of the various tissues. Figure 1 shows that NEP activity in the P_3 fraction of normal liver from ACI/N strain rats was 21 ± 3 pmol/h/mg protein and it increased 56.6-fold in rapidly-growing hepatoma 3924A. By contrast, activity in the rapidly-growing normal livers from 7-day-old rats and in the

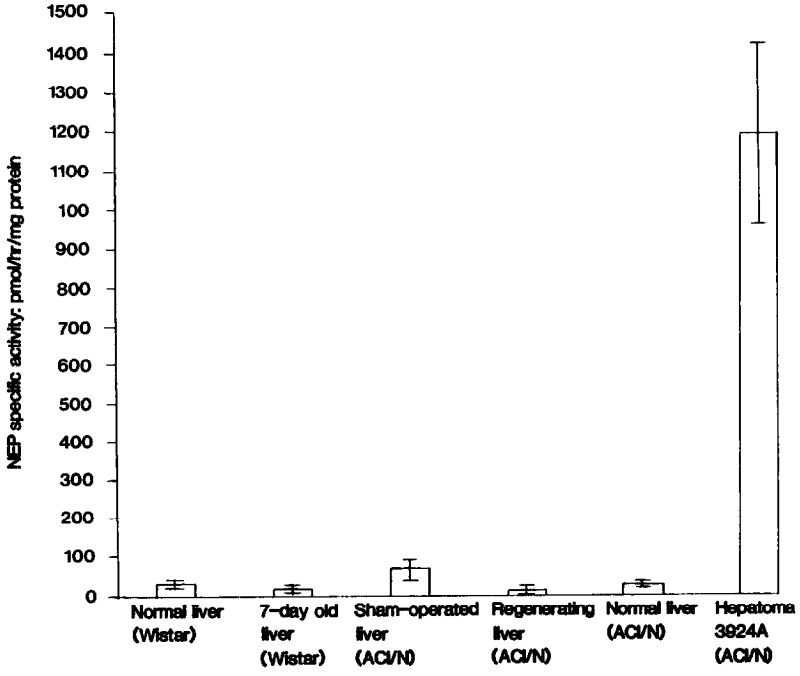

Fig. 1. Neutral endopeptidase 24.11 activity in P_3 fractions of normal and neoplastic livers. The data are given as means ± S.E.

regenerating liver decreased to 39 and 8% of that of the corresponding control livers. Thus, the strikingly increased NEP activity in the hepatoma was specific to neoplastic transformation and it was not due to rapid cell proliferation (regenerating liver) or differentiation (7-day-old rat liver).

To assure that the enzyme activity measured was authentic NEP, only activity which was completely inhibited by 1 uM phosphoramidon, a specific inhibitor of NEP, was taken into consideration. In addition, immunoprecipitation was carried out with polyclonal rabbit antiserum to human NEP. This polyclonal antiserum cross-reacts well with rat NEP. The amino acid sequence of NEP is very well preserved. For example, of the 742 amino acids in human NEP, 42 are different in the rat, but only six of them are non-conservative changes (22,23). For these reasons we attempted to immunoprecipitate the rat hepatoma NEP with our polyclonal antiserum, an additional proof for the presence of authentic NEP in tumor cells. Indeed, incubating the solubilized NEP from the hepatoma with antiserum, followed by precipitation with Protein A, resulted in the loss of 57 ± 0.4% of the

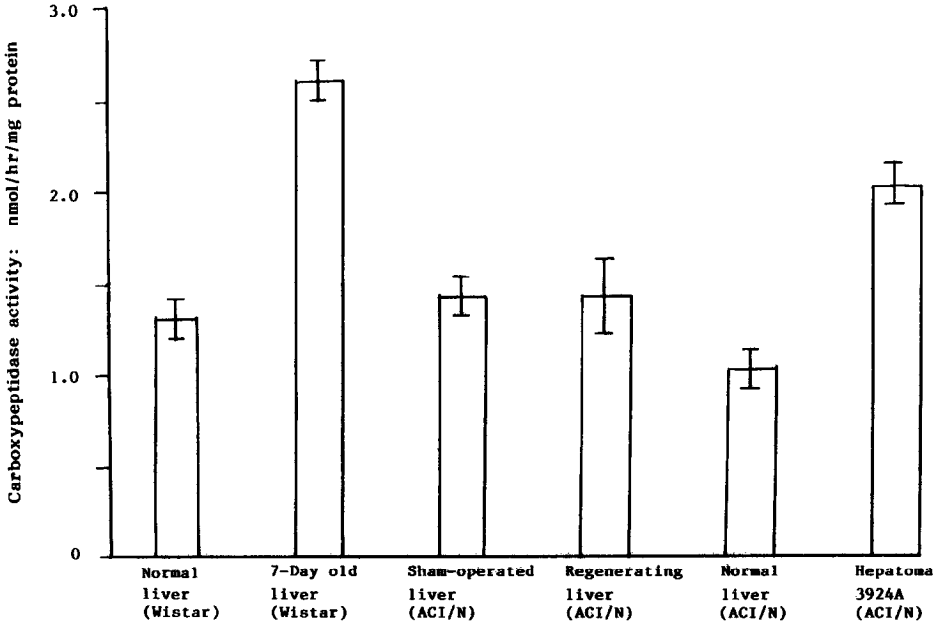

Fig. 2. Carboxypeptidase M activity in P_3 fractions of normal and neoplastic livers. The data are given as means ± S.E.

activity. In contrast, incubation with normal preimmunized rabbit serum reduced the activity of NEP by only 2.7 ± 0.8%.

CPM Activity. NEP is a transmembrane peptidase with the N-terminal 26 amino acids inserted in the bilayer of the plasma membrane of cells (22,24). To determine whether the increase in NEP activity could be due to a general elevation in activities of plasma membrane enzymes, we assayed another plasma membrane enzyme CPM (19). This enzyme is attached to cell membrane at its C-terminal end (25) in a great variety of cells. Figure 2 shows the distribution of CPM activity in the P_3 fractions of various tissues. It is clear that the pattern of activity differs completely from that of NEP measured in the same tissues (Fig. 1). There is at most a two-fold variation in the membrane carboxypeptidase activity in the extracts, including the hepatoma (Fig. 2).

The selective elevated expression of NEP in solid hepatoma 3924A is of interest as a marker of neoplastic transformation. However, the function of NEP in these tumor cells has not yet been determined. Among the best-known substrates that NEP can cleave under in vivo or in situ conditions are enkephalins (1,6), substance P (26), bradykinin (1,27), F-Met-Leu-Phe (1,27) and the atrianatriuretic peptide (1). It is possible that one of these peptides is linked with cell proliferation in a way which is still not understood. A plausible theory might be based on the assumption that NEP may activate an extracellular growth factor for the hepatomas or inactivate cell proliferation-inhibitory factors. The strikingly increased NEP activity in the rapidly growing hepatoma is specific to these transformed cells because no similar change occurred in rapidly growing differentiating or regenerating liver. The increased NEP activity may confer selective advantages to the cancer cells.

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