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Serum Enkephalin-degrading Aminopeptidase Activity in N-Methyl Nitrosourea-induced Rat Breast Cancer

MARIA PILAR CARRERA[1], MARIA JESUS RAMIREZ-EXPOSITO[1], MARIA TERESA VALENZUELA[2], BASILIO DUENAS[3], MARIA JESUS GARCIA[1], MARIA DOLORES MAYAS[1] and JOSE MANUEL MARTINEZ-MARTOS[1]

[1]Departamento de Ciencias de la Salud, Area de Fisiologıa, Universidad de Jaen, Jaen; [2]Distrito Sanitario Granada Nordeste, Avda. Mariana Pineda, s/n, Guadix, Granada; [3]Unidad de Patologıa Mamaria, Complejo Hospitalario de Jaen, Jaen, Spain

Abstract. *Background: Breast cancer is the most frequent spontaneous malignancy diagnosed in women in the western world, although no specific etiological agent(s) or the mechanism responsible for the initiation of the disease has been identified as yet. Enkephalins (Leu[5]-enkephalin and Met[5]-enkephalin) (ENK) act in the breast in different ways such as modulating esteroid receptors and proteases secretion. ENK are hydrolyzed by specific enzymes, leading to their inactivation, such as the enkephalin-degrading tyrosyl aminopeptidase (EDA). Breast tumours induced in rats by administration of N-methyl-nitrosourea (NMU) constitute a useful tool for dissecting the multistep process of carcinogenesis, which involves initiation, promotion and progression. The aim of the present work was to analyse EDA activity (E.C: 3.4.11.-) in serum of rats with mammary tumours induced by NMU, to evaluate the potential value of this activity as a biological marker of the carcinogenesis process, and the putative role of ENK in the promotion and progression of the disease. Materials and Methods: Tumours were induced by intraperitoneal injection of three doses of NMU at 50, 80 and 110 days after birth. Serum EDA was measured fluorimetrically using tyrosyl-β-naphthylamide as substrate. Results: The increase found in EDA activity suggests the existence of decreased serum circulating levels of ENK in rat with mammary tumours induced by NMU. Conclusion: Although the exact role of ENK in breast cancer initiation, promotion and/or progression remains unknown, our results suggest that changes in EDA activity might play an important role in the origin and evolution of breast cancer.*

Correspondence to: Dr. Jose Manuel Martınez-Martos, Área de Fisiologıa, Facultad de Ciencias Experimentales y de la Salud, Universidad de Jaen, Campus Universitario 'Las Lagunillas', E-23071, Jaén, Espana. Tel: +34 953 212 600, Fax: + 34 953 212 141, e-mail: jmmartos@ujaen.es

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Breast cancer, the most frequent spontaneous malignancy diagnosed in women in the western world, is continuously increasing in incidence in industrialized nations. Although breast cancer develops in women as the result of a combination of external and endogenous factors such as exposure to ionizing radiation, diet, socioeconomic status, and endocrine, familial, or genetic factors, no specific etiological agent(s) or the mechanism responsible for the initiation of the disease has been identified as yet (1,2). Enkephalins (Leu[5]-enkephalin and Met[5]-enkephalin) (ENK) act in the breast in different ways such as modulating esteroid receptors, proteases secretion and through interaction with cytoskeletal elements (3). It has been assumed that ENK are hydrolyzed by specific enzymes, leading to their inactivation. To date, most information about ENK degradation has been described in brain tissue. Two enzymatic pathways are considered to be of great importance for the degradation of ENK (4). These are the hydrolysis of the Gly-Phe bound by the membrane-bound enzyme neprilysin (5) and the breakdown of the Tyr-Gly bond by the enkephalin-degrading tyrosyl aminopeptidase (EDA) (6).

Breast tumours induced in rats by administration of N-methyl-nitrosourea (NMU) constitute a useful tool for dissecting the multistep process of carcinogenesis, which involves initiation, promotion and progression (7). The major attributes of this model include the fact that the proportion of mammary carcinomas that are ovarian hormone-dependent is similar to that observed in the human disease; that the carcinomas induced are aggressive and locally invasive; and that there is a clear operational distinction between the initiation and promotion stages of the disease process (8-12).

The aim of the present work was to analyse EDA activity (E.C: 3.4.11.-) in serum of rats with mammary tumours

Table I. *Values of specific enkephalin-degrading aminopeptidase (EDA) activity in serum of control and N-methyl-nitrosourea (NMU)-treated rats. Results are expressed in picomoles of tyrosyl-β-naphthylamide hydrolysed per min and per mg of protein.*

Rat #	Control	NMU
1	34.49	68.68
2	60.68	100.94
3	43.66	201.045
4	68.96	85.15
5	78.58	106.61
6	103.57	91.56
7	102.53	104.88
8	49.67	145.39
9	41.88	120.81
Mean ± SEM	64.89 ± 8.55	113.89 ± 13.073[a]

[a]Significant at $p<0.01$

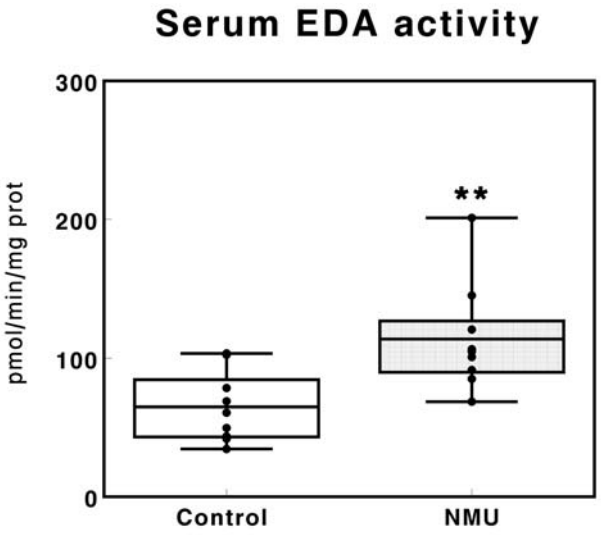

Figure 1. *Box-Whisker graph of specific enkephalin-degrading aminopeptidase (EDA) activity in serum of control and N-methyl-nitrosourea (NMU)-treated rats. Result are expressed in picomoles of tyrosyl-β-naphthylamide hydrolysed per min and per mg of protein (Mean ± SEM; n= 9; **p<0.01).*

induced by NMU, to evaluate the potential value of this activity as a biological marker of the carcinogenesis process and the putative role of ENK in the promotion and progression of the disease.

Materials and Methods

Animals and treatment. Forty female virgin Wistar rats (164.7 ± 4.7 g body weight) were used in this work. The animals were provided from the animal house of the University of Jaén, Spain, and maintained in an environment controlled under constant temperature (25˚C) with a 12h-light / 12h-dark cycle. All animals were allowed access to water and food *ad libitum*. The experimental procedures for animals use and care were in accordance with the European Community Council directive (86/609/EEC). The rats were randomly divided into two groups. One group were injected intraperitoneally with three doses of 50 mg/Kg body weight of NMU dissolved in distilled water (10 mg/ml) at 50, 80 and 110 days after birth, as described by Rivera *et al.* (13). Tumours induced by this method are oestrogen-dependent. All rats were at estrus at the first NMU injection, verified by daily vaginal smears. The control group received the vehicle only. For tumour detection and growth control, rats were examined by palpation 2 days each week after the second NMU injection. The number of tumours were recorded, and the major and minor diameters of each tumour were measured with a caliper. Body weight was determined periodically every week. Other tumour growth parameters were also determined: latency period, defined as the days between the first NMU injection and the appearance of the first tumour; tumour incidence, defined as the percentage of rats that developed at least one tumour; and mean tumour number per rat (n/t), defined as the number of tumours per rat in animals developing at least one tumour. One hundred and twenty-two days after the first NMU injection, the animals were sacrificed under equithensin anaesthesia (2 ml/kg body weight). Blood samples were obtained through the left cardiac ventricle and centrifuged for 10 minutes at 3000g to obtain the serum. These samples were frozen and stored at -80˚C, until use.

Serum enkephalin-degrading aminopeptidase activity (EDA) assay. Serum EDA was measured fluorimetrically using tyrosyl-β-naphthylamide (TyrNNap) as substrate. Briefly, 10 μL of each sample were incubated in triplicate for 30 minutes at 37˚C with 100 μL of the substrate solution containing 100 μM of TyrNNap and 0.65 mM dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 7.4.

All the reactions were stopped by adding 100 μl of 0.1M acetate buffer, pH 4.2. The amount of β-naphthylamine released as the result of the enzymatic activity was measured fluorimetrically at 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were also quantified in triplicate by the method of Bradford, using bovine serum albumin (BSA) as standard.

Statistical analysis. To analyse the differences between control group and the animals with mammary tumours induced by NMU injections, we used the unpaired Student's *t*-test. All comparisons with *p* values below 0.05 were considered significant.

Results

Tumour growth parameters in rats showed a latency period (Mean ± SEM) of 113.0 ± 4.2 days between the first NMU injection and the appearance of the first tumour, with a 60% tumour incidence. The mean tumour number per rat (Mean ± SEM) was 1.93 ± 0.4 tumours. The specific EDA activity in serum of controls and NMU-treated rats is shown in Table I and Figure 1. Serum EDA activity increased significantly ($p<0.01$) by 75.5% in NMU-treated rats.

Discussion

To our knowledge, the present work reports, for the first time in the literature, changes in serum EDA activity in carcinogenesis, using an animal model of breast cancer induced by NMU. Our results showed an increase in EDA activity in the serum of NMU-treated rats, suggesting the existence of decreased circulating levels of ENK. It is well described in many cancer cell lines, including breast, prostate, lung, brain, head and neck, retina and the gastrointestinal tract, that opioids decrease cell proliferation in a dose-dependent and reversible manner (14-20). Therefore, the lack of ENK due to the increase of activity of their degrading peptidase (EDA) could be responsible, at least in part, for breast cancer progression.

The breast is a hormone-responsive gland *par excellence*. Its development is influenced by hormones and growth factors, responding selectively to given hormonal stimuli with either cell proliferation or differentiation (21-23). Breast cancer, which is the most common neoplastic disease in females, continues to rise in incidence. The failure to eradicate this disease is largely due to the lack of identification of a specific etiological agent, the precise time of initiation and the molecular mechanisms responsible for cancer initiation and progression (7). Therefore, the role of ENK/EDA on breast cancer could be of great interest not only for use as a serum biological marker, but also as an important target of drug action.

Several laboratories have reported that ENK act as growth factors in neural and non-neural cells and tissues, in addition to serving for neurotransmitter /neuromodulation in the nervous system. Met^5-enkephalin, also known as native opioid growth factor (OGF), has been identified as a negative growth regulator (18-19,24-26). Met^5-enkephalin is broad-based in action and functions in development, cancer, cellular renewal, wound healing and angiogenesis (27).The biological effects of Met^5-enkephalin can be blocked by naloxone and are stereospecific, pharmacological characteristics suggesting involvement of an opioid receptor (28,29) with different characteristics from classic opioid receptors (28). Assuming that the peptide and receptor are in a delicate equilibrium, alteration in one or both peptide and receptor could have a profound effect on growth, and EDA activity is one of the main regulators of circulating levels of Met^5-enkephalin.

On the other hand, Maneckjee and coworkers (30) have described that opioids significantly inhibited the growth of the human breast cancer cell line MCF-7 in a dose-dependent manner when grown in the presence of 10% fetal bovine serum, but this inhibitory effect was reversed by the simultaneous administration of the opioid receptor antagonist, naloxone. However, the opioid effect appears to be restricted to the hormonally-responsive fraction of MCF-7 cell growth. In fact, cells grown in the presence of charcoal-stripped fetal bovine serum are refractory to the effects of the opioids unless the media is supplemented with estradiol. These researchers also postulate an important regulatory role for opioids in the growth and development of human breast cancer. These results were later corroborated by Panagiotou and coworkers (31), who reported that only the steroid-hormone-sensitive human breast cancer cell lines MCF-7 and T47D respond to opioid growth inhibition in a dose-dependent manner, postulating the existence of an interaction between the opioid and steroid receptor system. In fact, we have also recently described the decrease of pyrrolidon carboxypeptidase activity in serum of rats with mammary tumours induced by NMU (32), which points out high circulating levels of its substrate gonadotropin releasing hormone (GnRH). Increased levels of GnRH lead to increased levels of gonadal steroid hormones (33). Furthermore, the method of induction of tumours used in this study has been reported as estrogen-dependent (13). The peptide oxytocin also seems to be involved (34).

Opioids participate in the regulation of hypothalamus-pituitary-adrenal (HPA) axis activity under physiological conditions. This axis also has a strong influence on mammary carcinogenesis and we have demonstrated that gonadal steroid hormones also modified EDA activity at different levels of the axis (35,36).

To conclude, although the exact role of ENK in breast cancer initiation, promotion and/or progression remains unknown, our results suggest that changes in EDA activity might play an important role in the origin and evolution of breast cancer. Furthermore, serum EDA activity could be a useful serum marker to rapidly predict the sensitivity of a tumour to therapy, the maintenance or remission or an eventual occult disease, which might permit a better monitoring of cancer and rapid selection of more effective therapeutic/experimental means.

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