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Met-enkephalin induces cytolytic processes of apoptotic type in K562 human erythroid leukemia cells

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Abstract Cytolytic activity of Met-enkephalin, an endogenous opioid peptide, was studied within the 10^{-7}–10^{-17} M concentration range in K562 human erythroid leukemia cells. Cytolytic activity was determined by the trypan blue inclusion method after 13, 15 and 18 h of Met-enkephalin co-incubation with target cells. Discrete maxima of cytolytic activity were detected at concentrations of 10^{-9}–10^{-10}, 10^{-13} and 10^{-15} M. Cytolysis was accompanied by internucleosomal DNA fragmentation which is indicative of the mechanism of cell death being apoptosis.

Key words: Met-enkephalin; Cytotoxicity; Apoptosis

1. Introduction

Morphine has recently been shown to induce apoptosis in human lung cancer cells [1], the fact suggesting involvement of opioid ligands in signal-induced tumour cell death. A wide spectrum of both normal [2] and transformed cells [3] are known to bind opioid peptides. In particular, K562 cells have been shown to bind enkephalins and to release enzymes capable of hydrolysing these peptides [3]. Therefore, we studied cytolytic activity of Met-enkephalin, an endogenous ligand of the opioid receptor system in K562 tumour cells.

2. Materials and methods

2.1. Cell culturing

K562 (human erythroid leukemia) cells were cultured as described in [4]. Briefly, the cells were generated in RPMI 1640 medium enriched with 5% fetal calf serum (Gibco BRL) and supplemented with 2 mM of glutamate, 100 U/ml of penicillin and 100 mg/ml of streptomycin (Gibco).

2.2. Cytotoxicity assay

K562 and L929 cells (5×10^{-4}/well) were placed into 94 well plates (Flow) and co-incubated with 100 μl of serum-free RPMI 1640 medium containing different concentrations of Met-enkephalin for 13 h. Untreated cells co-incubated with serum-free medium for the same time interval were used as negative control. K562 cells were pelleted for 5 min at $300\times g$, then 60 μl of supernatant were removed and the remaining cells were stained directly in culture medium. Cytotoxicity of the substance Met-enkephalin was determined as the percentage of trypan blue that stained cells with damaged plasma membranes, according to Eq. 1:

$$\text{Cytotoxicity}(\%) = ((\text{stained cell number in the sample}) : (\text{total cell number in the sample}) - (\text{stained cell number in the negative control}) : (\text{total cell number in the negative control})) \times 100\% \quad (1)$$

More than 200 cells were examined in each well. The experiments where percent of dead cells in control wells was more than 15% have been excluded.

2.3. DNA fragmentation assay

DNA fragmentation was analysed by electrophoresis in 1% agarose (Pharmacia) gel as described in [5].

K562 cells (10^6/well) were placed into 24 well plates (Flow) and co-incubated with 300 μl of serum-free RPMI 1640 medium containing different concentrations of Met-enkephalin for 13 h. Untreated cells co-incubated with serum-free medium were used as negative control.

The cells were then treated with lysis buffer (10 mM Tris-HCl, 20 mM EDTA, 0.2% Triton X-100) and the nuclei and cell debris were pelleted. The DNA in the postnuclear supernatant was precipitated by isopropanol and separated in 1% agarose gel. DNA fragments were stained using ethidium bromide. DNA fragmentation was studied in four separate experiments.

2.4. Statistical analysis

Statistical analysis of the data obtained was carried out by Student's *t*-test. Three parallel wells containing the same concentrations of cytolytic substance and three control wells were examined in each separate experiment. The reproducibility of raw data was tested in 11 separate experiments. The absolute cytotoxicity values (%) were determined as stained cell number versus total cell number ratio (Eq. 2). The reliability (statistical significance) of the differences obtained in control and experimental wells was estimated by Student's *t*-test for $P < 0.05$ and $P < 0.01$ as described in [6].

The cytotoxicity values determined in each experiment were determined according to Eq. 1. The mean values, standard deviations (S.D.) and coefficient of variation (CV) were determined. CV values were determined for each concentration of Met-enkephalin according to the formula: [Mean value] : [S.D.] × 100%. The values with CV not more than 30% were assumed as reliable according to [6].

3. Results and discussion

The analysis of cytotolytic activity mediated by Met-enkephalin was carried out with K562 human erythroid leukemia cell line. The peptide in the 10^{-7}–10^{-16} M concentration range was incubated with tumor cells for 13 h. The percent of dead cells was determined by use of the trypan blue inclusion test and compared with negative (untreated cells) control. Under these conditions, Met-enkephalin was cytotoxic to K562 cells in the 10^{-7}–10^{-16} M concentration range. As seen from Fig. 1, three discrete maxima of cytotoxicity were detected at 10^{-9}–10^{-10}, 10^{-13} and 10^{-15} M concentrations of the peptide. As seen from Table 1, statistical evaluation of the data obtained allowed us to assume the determined cytotoxicity values as reliable.

DNA fragmentation associated with corresponding maxima of cytotoxicity was analysed by agarose gel electrophoresis. As seen from Fig. 2 Met-enkephalin induces internucleosomal DNA fragmentation in K562 cells at 10^{-10} and 10^{-13}–10^{-15} M concentrations corresponding to the maximal values of cytolytic activity. One of the most distinguishing features of apoptosis is the disintegration of chromatin within the nucleus

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Abbreviations: TNF, tumour necrosis factor; NT II, neurotoxin II from *Naja naja oxiana* cobra venom

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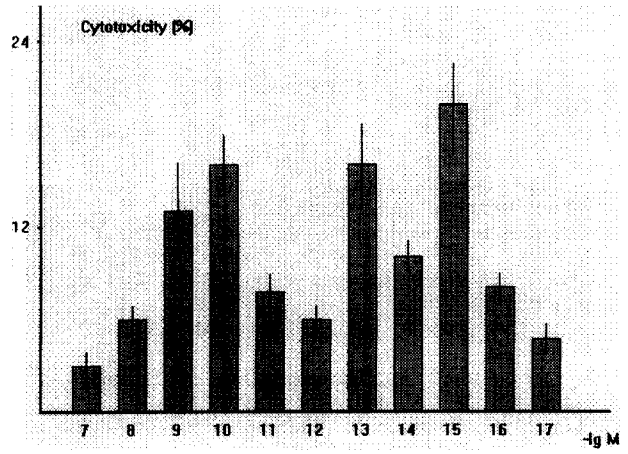

Fig. 1. Cytolytic processes mediated by Met-enkephalin in K562 cells at hour 13.

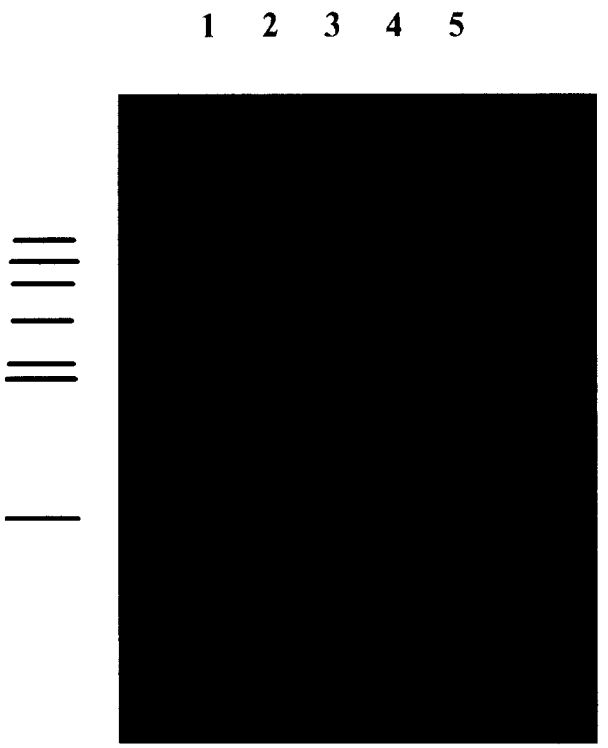

Fig. 2. DNA fragmentation induced by Met-enkephalin at hour 13. λ *Hin*dIII standards were used as molecular weight markers. Lanes: 1, control; 2, 10^{-10} M; 3, 10^{-13}; 4, 10^{-14}; 5, 10^{-15} M Met-enkephalin.

leading to the formation of 200 bp DNA fragments. As described by Golstein et al. [7], the presence of internucleosomal DNA fragmentation has been used as a major marker for cell death of this type [7,8] and we may thus conclude that Met-enkephalin induces the apoptotic mechanism of cell death in K562 tumor cells.

Several aspects of the results obtained require further comment and explanation. First, the observed phenomenon is not restricted to Met-enkephalin. Similar effects were also exerted by acetylcholine receptor antagonists: neurotoxin II [9], tubocurarin [4] and some endogenous cytolytically active substances, including TNF and substance P [10].

The non-saturable character of the cytolytic process was also observed by means of other methods based on vital dye staining. In particular, comparable results were obtained for *Fas*-ligand [11] and hepatocyte growth factor [12]. These data also demonstrate that specifically induced cytolysis may be associated with the development of several complex processes.

Several explanations for this phenomenon can be suggested. First, the asynchronous cell culture contains cells passing simultaneously through different stages of the cell cycle. Some of these stages may be more sensitive to cytolytic action whilst others are relatively resistant [13]. Therefore, the interaction of a cytolytic substance with cells differing in their cell cycle phase may lead to a variety of cytotoxic, cytostatic or proliferative effects [14]. Second, this phenomenon may be also caused by the involvement of several receptor subtypes associated with different signal transduction systems as suggested by Clement and Stamenkovic [11]. In this case, the different concentration of cytolytic substance may also induce a variety of effects, including both induction and inhibition of cytolysis. Third, the target cells of the same culture may also differ in the expression level of intracellular factors involved in regulation of cell death processes. In this case, the different subpopulations of target cells may react differently to the same signal. At the same time, several factors of this kind may contribute simultaneously to developing cytolysis inducing a number of cell death patterns.

The low concentrations of Met-enkephalin required for induction of cytolysis indicate the high specificity of this effect. Met-enkephalin induced apoptosis at concentrations significantly lower than its binding constant (about 10^{-10} M) as detected by Scatchard's radio-ligand method [15]. In contrast, morphine was cytotoxic in the 10^{-6}–10^{-7} M concentration range [1]. It was demonstrated earlier that the association of radiolabelled Leu-enkephalin with K562 cells was saturable at 10^{-14}–10^{-15} M and that the binding dynamics is quite complex, suggesting the involvement of several association mechanisms [3]. The kinetic parameters of Leu-enkephalin binding correlate closely with the characteristics of Met-enkephalin-mediated cytolytic effects as studied on this cell line. The highly specific action of the endogenous opioid peptide, Met-enkephalin, demonstrates the involvement of opioid receptors in induction of tumour cell death processes. The

Table 1
Statistical significance and reproducibility of cytotoxicity values represented in Fig. 1

Statistical parameters	Concentration of Met-enkephalin (M)										
	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}	10^{-15}	10^{-16}	10^{-17}
Mean values	3	6^a	13^b	16^b	8^a	6^a	16^b	10^b	20^b	8^a	5
Standard deviation	1	1	3	2	1	1	3	1	3	1	1
CV (%)	33	17	23	13	13	17	19	10	13	13	20

Statistical significance of cytotoxicity values is indicated as $^aP < 0.05$ and $^bP < 0.01$.

presence of more than one process also supports the possibility of the involvement of different subtypes of opioid receptors [16] in cytolysis. Opioid peptides, in particular, Met-enkephalin, were demonstrated to exert growth inhibitory activity on various tumour types [17,18]. The presence of Met-enkephalin and its precursor, proenkephalin A, was demonstrated in a wide range of brain tumours. A significant inverse correlation between the Met-enkephalin level and degree of malignancy was found for several tumour types [19]. Thus, it may be suggested that the opioid system is involved in autocrine inhibition of tumour development. At the same time, Met-enkephalin-containing peptides were shown to be produced by activated thymocytes and to regulate lymphocyte proliferation [20]. It can be proposed that endogenous opioid receptor ligands also participate in tumour cell cytolysis mediated, in particular, by cytotoxic lymphocytes.

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