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GENOTOXIC EVALUATION OF [DOTA, Tyr^3]OCTREOTATE LABELED WITH ^{131}I AND ^{177}Lu IN HUMAN PERIPHERAL LYMPHOCYTES *IN VITRO* BY MICRONUCLEUS ASSAY

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ABSTRACT

The radiolabeled receptor-binding peptides have being used for cancer diagnosis and therapy. The octreotate, a somatostatin analogue peptide, bound to various tumors expressing sst receptors (thyroid, pancreas, prostrate, melanoma and lymphomas). The amount and the type of receptors for somatostatin influence the tissue uptake. The [DOTA, Tyr^3]octreotate has been used because of its high affinity to somatostatin subtype receptors $sstr_2$ and $sstr_5$. The pharmacokinetic study showed that the blood clearance is rapid and only 9% of the intravenous injected activity remains in human blood after one hour. The aim of this study was to evaluate the cytogenetic effect of radiolabeled [DOTA, Tyr^3]octreotate in blood cells *in vitro*, using the cytokinesis-block micronucleus (MN) assay. This technique allows evaluating the mutagenic effects of both endogenous and exogenous agents at chromosome level. Blood samples of healthy donors were collected in heparinized syringes and exposed to different activities of [DOTA, Tyr^3]octreotate labeled with with ^{131}I (n=3) and ^{177}Lu (n=3), where radioactive concentration ranged from 600 to 5600 kBq/mL, corresponding to an injected activity of 3.1 to 28.9 GBq in a reference man of 70 kg weight. ^{131}I and ^{177}Lu are beta- and gamma-emitters. After one-hour exposition to radiopharmaceuticals at 37°C, the cells were washed with culture medium for removing the non internalised octreotate and cultivated for 72 hours, according to criteria adopted by the IAEA. The results showed a positive correlation between radioactive concentrations (X) and the frequency of binucleated cells with micronuclei (Y) ($P<0.05$). The model for the best fit of data was the linear one ($Y = a + bX$). The equation for [^{131}I-DOTA, Tyr^3]octreotate was $Y = (1.634 \pm 0.236) + (0.912 \pm 0.137)\ 10^{-3}\ X$ and for [^{177}Lu-DOTA, Tyr^3]octreotate was $Y = (1.715 \pm 0.342) + (0.743 \pm 0.135)\ 10^{-3}\ X$. The non labeled molecule, [DOTA, Tyr^3]octreotate, has no influence in the induction of cytogenetic damage. The micronucleus assay with rat pancreatic tumor cells (AR42J) that express the $sstr_2$ receptor for somatostatin, submitted to these radiopharmaceuticals, are in course.

1. INTRODUCTION

The use of peptides as a means of targeting radionuclides to specific tissues has recently become a promising tool in both diagnostic and therapeutic applications. The choice of these peptides, natives or synthetics, has been based, fundamentally, in favorable biological characteristics, such as rapid blood clearance, low toxicity, high uptake into tumor tissue and low immunogenicity [1,2].

Among various peptides, the octreotide, a somatostatin analog, consisting in an 8 amino acids residues, has received special attention as a consequence of the identification of somatostatin receptors in different kinds of tumors such as neuroendocrine, central nervous system, breast, lung, pancreas, thyroid, colon and lymphatic tissue tumors [3,4,5,6,7,8,9,10]. The octreotide

has been used either for localization of primary tumors as for metastatic ones. The amount and the types of somatostatin receptors ($sstr_1$- $sstr_5$) that cell expresses will influence the uptake of the radiopharmaceutical [2].

The somatostatin is a small neuropeptide that exists in at least two circulating forms: as 14- and 28-amino acid peptides, that is widely distributed throughout the body and has numerous functions: it inhibits the growth hormone secretion by pituitary and also inhibits hormone secretion from the gastrointestinal tract, whereas in the nervous system it functions as a neurotransmitter besides a role in the immune system as well [11].

The radionuclides used to label the octreotide were ^{111}In [6], ^{90}Y [2,12,13,14], ^{177}Lu [15,16], ^{131}I [17] and ^{123}I [18,19,20], which were coupled to the chelators (DTPA – diethylenetriaminepentaacetic acid, TETA – tetraazacyclotetradecanetetraacetic acid or DOTA - tetraazacyclododecanetetraacetic acid) forming a complex biologically stable. The choice of these radionuclides is related, principally, to their energy and short tissue penetration range [15,21].

Recent studies have shown that the somatostatin analogue [DOTA^0, Tyr^3]octreotate (DOTATATE), in which the C-terminal threoninol is replaced by threonine [9], presents a higher affinity for the somatostatin receptor subtype 2 (about nine-fold increase) compared with [DOTA, Tyr^3]octreotide (DOTATOC), both *in vitro* and *in vivo* in animal model [22]. Besides, octreotate labeled with the beta- (E_{max} 495 keV, 78%) and gamma-emitting (208 keV, 11%) radionuclide ^{177}Lu [23,24] was reported to have a very successful impact on tumor regression and animals survival [15,24].

On the basis of the above considerations, the aim of this study was to evaluate and compare the cytogenetic effect of [^{131}I DOTA^0, Tyr^3]octreotate and [^{177}Lu DOTA^0, Tyr^3]octreotate, using the micronucleus assay in peripheral blood lymphocytes of healthy donors *in vitro*. There are no data in the literature about the cytogenetic effects associated to these radiopharmaceuticals labeled with ^{177}Lu and ^{131}I in human cells. The micronucleus assay is proposed as an alternative technique simpler and more rapid but equally sensitive to the chromosome aberration analysis [25]: it detects non-repaired damages, specifically to DNA double strand breaks [26].

2. MATERIAL AND METHODS

2.1. Donors

Venous blood was collected in heparinezed syringes from 6 healthy donors (26 ± 5 year old, both sexes). The blood samples were fractionated in plastic tubes containing 3.0 ml RPMI 1640 medium (Cultilab, Brazil) and exposed to different radioactive concentration of [^{131}I-DOTA, Tyr^3]octreotate and [^{177}Lu-DOTA, Tyr^3]octreotate, during one hour at 37°C. The radioactive concentration range was between 600 and 5600 kBq/mL. These concentrations correspond to an injected activity of 3.1 to 28.9 GBq in a reference man with 70kg weight. The incubation time of one hour was chosen considering the relatively rapid clearance of the radiopharmaceutical as only 9% of the injected activity remained in blood compartment [6].

For the evaluation of a possible influence of the non labeled molecule in induction of chromosome damage, the blood sample of one donor was incubated with [DOTA, Tyr^3]octreotate (1.7 μg/ml) and processed for the micronucleus analysis. The ethical committee of IPEN – CNEN/SP approved this study. All donors gave informed consent for the use of their blood cells.

2.2. Micronucleus assay

After the incubation time, samples were washed three times with RPMI 1640 medium and then set up to cytokinesis-block micronucleus assay [27]. The cells were cultivated in 4.0 ml RPMI 1640 medium (Cultilab, São Paulo, Brazil), 1.5 ml fetal-calf serum (Cultilab, São Paulo, Brazil), 100 μl phytohemagglutinin (Gibco BRL, Grand Island, USA) and incubated at 37°C for 72h. Cytochalasin B (Sigma, St. Louis, USA) at a final concentration of 6 μg/mL was added at 44h to block cytokinesis. At the end of incubation period, cells were harvested by centrifugation, given isotonic treatment and then fixed in a fresh fixative solution (methanol:acetic acid, 3:1). This fixation step was repeated twice and the pellet was resuspended in a small volume of fixative solution. Six to eight drops of each suspension were transferred onto microscope slides, air-dried and stained for 10 min with 5% Giemsa solution in phosphate Sorensen buffer. The frequency of MN was analyzed in a minimum of 500 binucleated cells (BNC) per sample. The number of mononucleated cells (MC) and multinucleated cells (MNC) was used for the calculation of the proliferation index: PI = ((MC + 2BNC + 3MNC)/total number of cells x 100). The values of PI were compared by one way ANOVA. Values of $p<0.05$ were considered significant. One experienced observer analyzed all slides with a light microscope under a 400x magnification. The binucleated cells were identified according to the criteria of Countryman and Heddle [28]: preserved cytoplasm, non-confluent, at the same focal plan, with diameters up to one-third of the main nucleus, having the same or a lighter color. Cells with more than 5 MN were not considered. Data from three independent assays were analyzed. The dose-response curves (frequency of binucleated cells with micronucleus versus radioactive concentration) were obtained by linear regression model, $Y = a + bX$, where a and b are model constants, Y the frequency of binucleated cells with micronucleus, and X the radioactive concentration. The slopes of the adjusted curves were compared with the Student's t-test. Values of $p<0.05$ were considered significant. The analyses were performed with the GraphPad Prism program (version 2.00).

3. RESULTS

The obtained cytogenetic data showed that there was a positive correlation ($p<0.05$) between the percentage of binucleated cells with micronucleus (Y) and the radioactive concentrations of radiopharmaceuticals (X) labeled with ^{131}I (Figure 1A) and with ^{177}Lu (Figure 1B). The best fit for these data was the linear model: $Y = (1.634 \pm 0.263) + (0.912 \pm 0.137)\ 10^{-3}\ X$ ($r = 0.9025$) for [^{131}I-DOTA, Tyr^3]octreotate and $Y = (1.715 \pm 0.342) + (0.743 \pm 0.135)\ 10^{-3}\ X$ ($r = 0.8563$) for [^{177}Lu-DOTA, Tyr^3]octreotate. Apparently, both types of radiopharmaceuticals induced similar chromosomal alterations at cytogenetic level.

The proliferation index varied between 1.13 and 1.59 at basal samples and from 1.08 and 1.53 for the cells exposed to either [^{131}I-DOTA, Tyr^3]octreotate or [^{177}Lu-DOTA, Tyr^3]octreotate. Thus, the proliferation index was not altered with the increase of the radioactive concentration of octreotate labeled either with ^{131}I (Figure 2A) or with ^{177}Lu (Figure 2B) in human peripheral lymphocytes (one way ANOVA, p>0.05).

It was possible to observe that the molecule [DOTA, Tyr^3]octreotate do not induced additional chromosome damages to blood lymphocytes (1.7% of binucleated cells with micronucleus) as well as any alteration in the cellular proliferation kinetics (proliferation index = 1.47) when compared to basal values (1.7% of binucleated cells with micronucleus and proliferation index of 1.51).

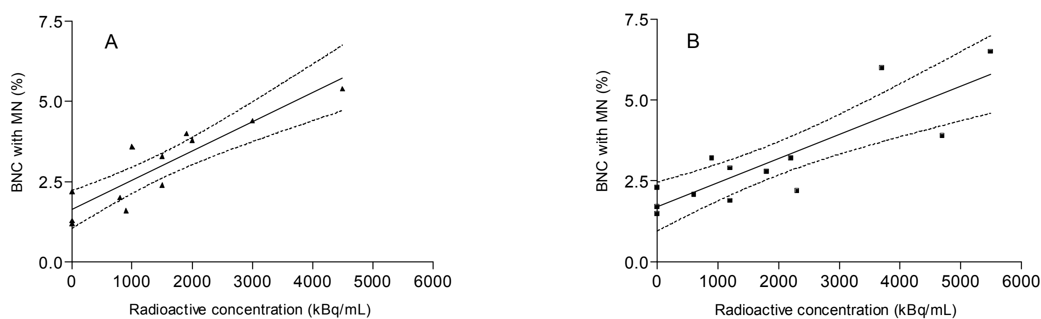

Figure 1. Dose-response curve for the percentage of binucleated cells (BNC) with micronucleus (MN) after one hour exposure of human peripheral lymphocytes to different radioactive concentrations of [^{131}I-DOTA, Tyr^3]octreotate (A) and [^{177}Lu-DOTA, Tyr^3]octreotate (B). The data were adjusted by linear regression: Y = (1.634 ± 0.263) + (0.912 ± 0.137) 10^{-3} X (r = 0.9025) for [^{131}I-DOTA, Tyr^3]octreotate and Y = (1.715 ± 0.342) + (0.743 ± 0.135) 10^{-3} X (r = 0.8563) for [^{177}Lu-DOTA, Tyr^3]octreotate.

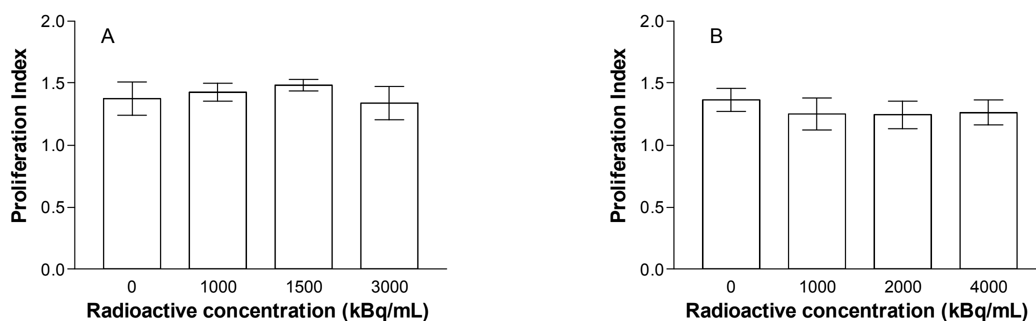

Figure 2. Proliferation index of human peripheral lymphocytes exposed for one hour to different radioactive concentrations of [^{131}I-DOTA, Tyr^3]octreotate (A) and [^{177}Lu-DOTA, Tyr^3]octreotate (B). No statistical differences were observed after the exposures (one way ANOVA, p > 0.05).

4. DISCUSSION

The synthesis and clinical application of the somatostatin analogue opened a new perspective in the identification and treatment of various tumors expressing somatostatin receptors [3,8,29].

The success of the therapeutic strategy relies, fundamentally, on the amount of radioactivity concentrated within tumor cells, which is determined by the rate of internalization of the radioligand and intracellular retention of the radionuclide [18].

In relation to other radiolabeled somatostatin analogues, [^{177}Lu-DOTA, Tyr^{3}]octreotate is considered a promising radiopharmaceutical for peptide receptor radionuclide radiotherapy because of the higher uptake in target tumor cells and the short tissue penetration (maximum range of 2 mm) which may be especially important for small tumors [15]. ^{177}Lu also emits gamma rays suitable for scintigraphy and subsequent dosimetry [16].

In the present study, the cytogenetic effects of [DOTA, Tyr^{3}]octreotate labeled with ^{131}I and ^{177}Lu were analyzed by micronucleus assay in human blood lymphocytes *in vitro*. These cells present high radiosensitivity, are easy to collect and are the first cells to enter in contact with the radiopharmaceutical, considering the endovenous administration via of the radiolabeled octreotate.

The cytogenetic analysis showed that the effects of [DOTA, Tyr^{3}]octreotate labeled with ^{131}I and ^{177}Lu were equivalent at cellular level and that [DOTA, Tyr^{3}]octreotate did not influence the induction of chromosome damage in human blood lymphocytes *in vitro*.

^{131}I is also beta- (E_{max} = 606 keV, 90%) and gamma-emitting (E = 365 and 637 keV, 10%) radionuclide with a physical half-life of 8.1 d that is proximal to that of ^{177}Lu (6.7 d). [^{131}I-DOTA, Tyr^{3}]octreotate can be considered an alternative radiopharmaceutical to [^{177}Lu-DOTA, Tyr^{3}]octreotate both of great interest in Nuclear Medicine for diagnostic and peptide receptor radionuclide therapy applications.

The next step of the present study is to analyze the effects of these two radiopharmaceuticals in rat pancreatic tumor cells (AR42J) that express the subtype 2 somatostatin receptors ($sstr_2$) [18].

5. CONCLUSIONS

The micronucleus assay showed that [DOTA, Tyr^{3}]octreotate labeled with ^{131}I and ^{177}Lu induced similar effects at chromosome level and did not interfere in the kinetic of cellular proliferation in human peripheral lymphocytes *in vitro*.

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