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Article

Synthesis and biological evaluation of ^{125}I-erythropoietin as a potential radiopharmaceutical agent for tumours

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Erythropoietin (EPO) is a glycoprotein hormone responsible for regulating erythropoiesis. Expression of EPO and EPO receptors (EPOr) has recently been demonstrated in some neoplastic cell lines and tumours, suggesting a potential new target for therapy. In this work, EPO was labeled with iodine-125 using the lactoperoxidase method, known to prevent damage to protein during radioiodination, and labeling conditions were optimized. *In vitro* stability studies have shown that ^{125}I-EPO is radiochemically stable for 20 days after radiolabeling. *In vitro* cell binding studies have demonstrated very low binding (<2%) of EPO to normal and neoplastic cell lines tested. As expected, the biodistribution in healthy mice exhibited comparatively high rates of fixation in the organs of the excretory system. Thyroid also proved to be a critical organ which may indicate *in vivo* dissociation of ^{125}I-EPO. In mice with induced melanoma, only a residual fixation in the tumour was evident. Further studies are warranted on other tumoral cell lines to better understand the binding process and internalization into cells. Studies on EPO labeled with carbon-11 could be valuable, since there is a greater chance of preserving the biological activity of the protein using this method.

Uniterms: Erythropoietin (EPO). Glycoprotein hormone. ^{125}I-erythropoietin/synthesis. ^{125}I-erythropoietin/biological evaluation. Erythropoiesis/regulation. Radiopharmaceuticals. Iodine-125/labelled. EPO expressive tumours.

A eritropoetina (EPO) é um hormônio glicoprotéico responsável pela regulação da eritropoese. Recentemente foi demonstrado que os receptores de EPO (EPOr) estão expressos em algumas linhas celulares neoplásicas, o que sugere a sua potencialidade como um novo alvo terapêutico. Neste trabalho a EPO foi radiomarcada com iodo-125 através do método da lactoperoxidase, menos agressivo para a viabilidade biológica das proteínas. A ^{125}I-EPO revelou ser radioquimicamente estável durante 20 dias após a síntese. Um estudo biológico *in vitro* em linhas celulares tumorais demonstrou que a ^{125}I-EPO apresenta uma ligação muito fraca (<2%), tanto em células normais como nas linhagens tumorais testadas. A biodistribuição em camundongos saudáveis apresentou taxas de fixação relativamente maiores nos órgãos excretores e a tireóide revelou ser o órgão crítico, o que pode indicar a dissociação *in vivo* da ^{125}I-EPO. No estudo em camundongos com melanoma induzido a fixação no tumor foi residual. Serão, no entanto, necessários novos estudos em outras linhagens tumorais para entender o seu processo de internalização e ligação nas células. Estudos da EPO radiomarcada com carbono-11 poderão também revelar-se interessantes, já que neste método há maior probabilidade da atividade biológica ser preservada.

Unitermos: Eritropoetina. Hormônio glicoprotéico. ^{125}I-erythropoietin/síntese. ^{125}I-erythropoietin/avaliação biológica. Eritropoese/regulação. Iodo-125/marcador. Tumores expressores de EPO.

INTRODUCTION

Due to the increasing incidence of neoplastic diseases in contemporary society, concerted efforts are being made in oncologic research, both in the area of therapy and diagnosis. The existing methods of therapy are most effective when the disease is detected early. For this reason, efforts are being made to develop diagnostic techniques that are increasingly sensitive and specific in order to detect any abnormal cell proliferation as early as possible. Nuclear Medicine has made a significant contri-

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bution toward this goal because it allows the detection of malignant proliferations on a molecular scale before the manifestation of physiological symptoms or detection by other conventional imaging techniques. To this end, the use of erythropoietin (EPO) is currently the focus of metabolically oriented studies aimed at the detection of tumours.

EPO is a glycoprotein hormone that regulates erythropoiesis. It acts in the advanced stages of the erythrocyte progenitor cells by promoting proliferation, maturation and apoptosis inhibition in order to decrease cell death rate in bone marrow (Fisher, 2003). The main production site of this hormone is located in the kidney, although it is also produced in smaller quantities in the liver in response to low oxygen concentration levels in the blood. Hypoxia is a key concept in the production of EPO. Several studies have shown a relationship between some cancers and the stimulation of EPO production due to hypoxia (Acs *et al.*, 2004a,b).

Many types of tumoral cells have a system of EPO autocrine production, which allows them to survive and proliferate under conditions of hypoxia (Lappin, 2003; Winter *et al.*, 2005). Thus, EPO is strongly involved in growth, viability and angiogenesis of a large number of tumours (Eccles *et al.*, 2003; Kumar *et al.*, 2005; Yasuda, Fujita, Matsuo, 2003). This means that these tumoral cells have EPO receptors (EPOr) on their surfaces that can be used as targets for the early detection of cancers by using EPO labeled with a radionuclide.

Use of immunohistochemical methods has yielded evidence proving that EPOr are present in breast cancer cells but absent in adjacent tissues (Acs *et al.*, 2002). This phenomenon seems to be a promising focus for future research directing chemotherapy agents to the EPOr of a tumour while sparing surrounding tissues (Lappin, 2003). This can also be important in Nuclear Medicine diagnosis or therapy through the labeling of EPO with iodine radioactive isotopes (^{131}I or ^{123}I) that are easily introduced into tyrosine, histidine and histamine residues in proteins.

EPO has a molecular weight of around 30kD, is composed of 165 amino acids, four carbohydrate groups (some with sialic acid termination) while one of its most important structural aspects is the fact that it has two disulfide bridges. One of these bridges ensures correct molecular form allowing binding to receptors. The breaking of this bridge leads to a loss of biological activity of the protein. Consequently, the introduction of an iodine atom in EPO is effected directly by electrophilic substitution, activated by electron-donor atoms (-OH, -NH_2), with the various carbohydrate groups containing sialic acid. Thus, radioiodination by electrophilic substitution using lactoperoxidase is the most appropriate method since it not only leads to higher specific activity, but is also a less aggressive method with a greater tendency to maintain the biological integrity of proteins (Murphy, 1976; Signore *et al.*, 1992).

MATERIAL AND METHODS

In this study, the oxidative radioiodination of EPO was performed enzymatically by the lactoperoxidase method given this is a less aggressive method of protein radiolabeling.

EPO solution was prepared in phosphate buffered saline (PBS) with pH 7.4 (0.16 g/L). A volume of 25 µL of this solution was mixed with 15 MBq of $Na^{125}I$, 4 µg of lactoperoxidase solution and 10 µL of H_2O_2 30%. The reaction mixture was stirred in a vortex and radioiodination took place at room temperature.

To optimize reaction time, five EPO radioiodinations were carried out under the same conditions. Each was interrupted by adding 150µl of sodium thiosulfate 10% after 2, 5, 10, 20 and 30 minutes, respectively. Radiolabeling efficiency was calculated using an optimized TLC-SG chromatographic method with ethanol, ether and water (5:5:2) where ^{125}I-EPO has an R_f of 0.0 and ^{125}I an R_f of 0.9.

The remaining free ^{125}I was separated from radioiodinated EPO by gel permeation chromatography (*Sephadex G-25*) with PBS at pH 7.4 as the eluent. The radioactivity of each fraction was measured using a gamma counter. The ^{125}I-EPO purified solution was kept at 6 ºC and an *in vitro* stability study was conducted for a 20-day period.

To analyze the binding rate of ^{125}I-EPO to normal and neoplastic cells, V79 (Chinese Hamster Lung Fibroblast Cell Line) and B16-F1 (epithelial cells of mice melanoma) cell lines were used, respectively. These lines were incubated for 3 hours in a medium containing ^{125}I-EPO (0.2 MBq/0.5 mL). For binding affinity control 0.5 mL of several modulator drugs were used: amiloride (1.3 mg/mL), chloroquine (2.6 mg/mL), quinacrine (2.3 mg/mL) and ouabain (3.6 mg/mL). Amiloride, chloroquine and quinacrine act as acidification inhibitors. Amiloride is also an inhibitor of Na^+/H^+ and Na^+/K^+ exchangers (Weiss, Lang, Bernhardt, 2004) while quinacrine acts on cationic channels causing modification of kinetic parameters and other effects by binding to DNA and inhibiting NADH oxidoreductase in membrane. Ouabain is an inhibitor of Na^+/K^+ ATPase and modifies membrane permeability to ions (Lodish *et al.*, 2001). After three hours had elapsed, cells were analysed by optical microscopy. Cells were subsequently dissolved in 0.5 mL of NaOH 0.1 N with 1% sodium dodecyl sulfate, and centrifuged to separate them from unbound ^{125}I-EPO. Finally, cells were controlled by a gamma counter.

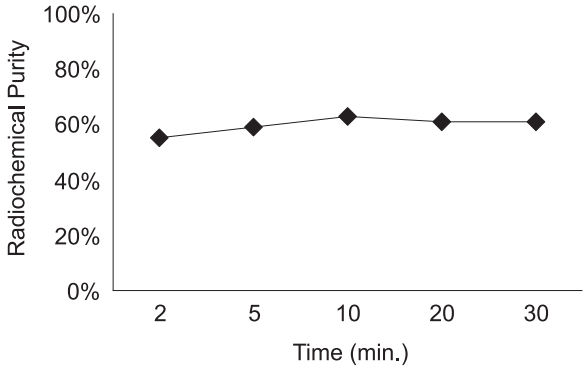

FIGURE 1 – Radiochemical purity of ^{125}I-EPO by radioiodination time.

^{125}I-EPO biodistribution was studied in mice in order to characterize its *in vivo* behavior, more specifically its fixation in several tissues, including tumoral tissue. This study was conducted in two phases using female Balb/c strain mice. In the first phase, the labeled and purified compound, with a radiochemical purity of 100%, was injected intraperitoneally (approximately 0.6MBq) into 8 healthy animals that were sacrificed by dislocation of the neck at two different time intervals: four of these animals were sacrificed 1 hour after injection, and the remaining mice, 4 hours after injection. In the second phase, the experiment was repeated in three female mice with a melanoma induced by subcutaneous injection of B16-F1 tumour cells. All of the mice were sacrificed by dislocation of the neck 4 hours after ^{125}I-EPO injection. Subsequently, the dissection of several organs of interest and neoplastic tissue was performed in order to measure their radioactivity using a dose calibrator. The biodistribution results were analysed according to Tukey's range test, with a value of $p<0.05$ considered significant. All the experiments were carried out in compliance with the guidelines for conduct in animal experimentation.

RESULTS AND DISCUSSION

Synthesis and stability of ^{125}I-EPO

By varying the duration of the radioiodination reactions of EPO and evaluating their radiochemical purities, a time period that tends to be more favorable for this procedure was identified. Through this experimentation it was concluded that the optimal labeling efficiency can be achieved after approximately 10 minutes of reaction (Figure 1). Thus, all subsequent radioiodinations of EPO were based on a standardized 10-minute reaction time which, associated with purification by gel permeation chromatography (*Sephadex G-25*), consistently yielded final products with radiochemical purities exceeding 99%.

Results of the *in vitro* stability study confirmed that the ^{125}I-EPO solution was stable for at least 20 days after radiolabeling and purification. On the sixth day after radioiodination, the compound exhibited a radiochemical purity of approximately 100%. At the end of the study, ^{125}I-EPO had a radiochemical purity of around 93%, thereby proving high stability in PBS at pH 7.4 stored at 6 ºC.

In vitro study with tumoral cell lines

In vitro studies of the biological viability of ^{125}I-EPO showed negligible binding affinity (<2%) for all cell lines tested, although this was slightly higher in V79 (Figure 2).

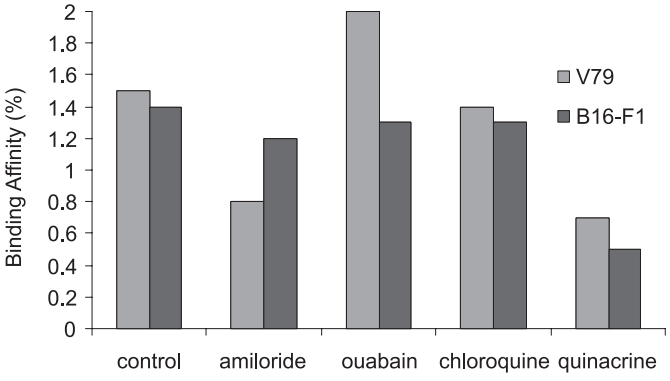

FIGURE 2 – Mean binding affinity of ^{125}I-EPO in V79 and B16-F1 cell lines.

With regard to different modifiers used, two of these seemed to have a negative effect on cell binding affinity. Quinacrine, which led to lower binding rates, proved to be cytotoxic for both types of cell lines at the concentration used. This effect was visible under optical microscope. Amiloride appeared to have an inhibiting effect on the binding affinity of ^{125}I-EPO in V79 cells, since it was the only modifier that presented higher binding percentages in B16-F1. Chloroquine showed a similar pattern to that seen in the control group, which may be indicative that this drug exerts no effect on the studied binding process. Finally, ouabain seemed to have a positive strengthening effect on V79 binding affinity.

Thus, this study demonstrates that ^{125}I-EPO had a weak binding affinity in the normal (V79) and tumoral (B16-F1) cell lines studied. These results may indicate that none of the tested cells expressed EPOr or that there may be a possible loss of biological activity caused by the incorporation of iodine in the molecule.

Biodistribution in animal model

After dissection of the mice, the injected dose (ID) per tissue (Eq. 1) and per gram of tissue (Eq. 2), were calculated for each animal.

$$\%\ ID\ \ per\ \ tissue = \frac{activity\ \ in\ the\ tissue}{total\ \ injected\ \ activity} \times 100 \qquad \text{Eq. 1}$$

$$\%\ ID\ \ per\ gram\ of\ \ tissue = \frac{\%\ ID\ \ per\ tissue}{weight\ of\ \ the\ tissue} \qquad \text{Eq. 2}$$

The fixation of radioactivity verified in mice sacrificed 4 hours after injection was shown to be lower than that in mice sacrificed after 1 hour (Figure 3A). This is because excretion increases with time after injection. The kidney and liver are the main routes of excretion and so have higher fixation rates. The levels of ^{125}I-EPO in the blood are also high because of slow clearance of EPO ($4h<T_{1/2}<11h$) (Kendall, 2001). The high radioactivity observed in thyroid, an organ that has a natural ability to capture iodine, suggests that, despite the observed *in vitro* stability, there may be some *in vivo* dissociation of ^{125}I-EPO, since the solution injected in the mice was almost completely free of iodine. The values obtained in the assessment of the mice with induced melanoma demonstrated a residual fixation of ^{125}I-EPO in tumour (Figure 3B) while other organs showed expected level of fixation, based on a previous study with a healthy animal model.

Owing to the suspected *in vivo* ^{125}I-EPO dissociation, a TLC-SG chromatography of the urine collected from one of the mice sacrificed 4 hours after injection was performed. Results revealed the presence of a radiochemical species with an R_f corresponding to iodine ($R_f = 0.9$). This further supported the theory of ^{125}I-EPO deiodination inside the body.

CONCLUSIONS

In this study, EPO radiolabeling with ^{125}I was performed to evaluate its potential as a diagnostic or therapeutic radiopharmaceutical in molecular imaging. This radioisotope of iodine was chosen for having the most appropriate characteristics for research purposes (low gamma emission energy, 35 keV, and a long half-life of 60 days) and because it mimics the use of ^{131}I or ^{123}I for radiotherapy or diagnostic radiopharmaceuticals, respectively.

The relative ease of radioiodination and purification of the molecule, the reproducibility of the process and its high stability, demonstrated by radiochemical purity as

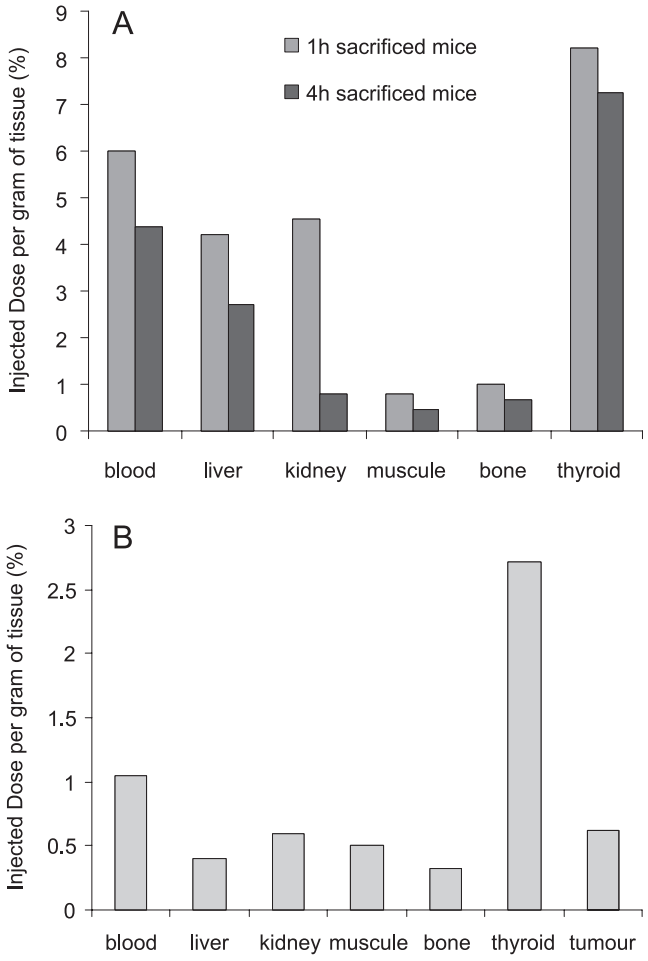

FIGURE 3 – Biodistribution of ^{125}I-EPO in mice. A. Comparative study in healthy mice; B. Study in 4h sacrificed mice with induced melanoma.

around 93% after 20 days of synthesis, are parameters that render ^{125}I-EPO a physicochemically desirable radiopharmaceutical for use in Nuclear Medicine laboratories.

The biological tests began with an *in vitro* study on normal and tumoral (V79 and B16-F1) cell lines, in an attempt to examine the binding mechanism of radioiodinated EPO. This procedure revealed that ^{125}I-EPO has a weak binding affinity to cells, since the binding values were very low (<2%). However, further studies on new cell lines are needed to confirm whether the weak binding affinity is due to the absence of specific receptors in the cells used or to the biological activity lost during EPO radioiodination.

Studying biodistribution in an animal model is an essential stage in the development of any radiopharmaceutical. The *in vivo* behavior of ^{125}I-EPO was investigated by intraperitoneal injection into healthy mice and animals with induced melanoma. The peritoneal cavity was chosen because it offers a greater absorption surface allowing faster entry into the bloodstream without the losses associated with

intravenous injection in the tail of the animals. The results in healthy mice showed an expected biodistribution pattern in which the organs involved in the excretion mechanism, such as liver and kidneys, had comparatively high fixation rates. Thyroid proved to be the critical organ, which may indicate the *in vivo* dissociation of ^{125}I-EPO. In the study involving mice with melanoma, induced by subcutaneous injection of B16-F1 tumour cells, the uptake of the radiation in neoplastic tissue was residual. This result should be expected since this same cell line showed very low levels of binding affinity in the *in vitro* study. Further biodistribution analysis in animals with different tumours, such as breast cancer, is now warranted. This is the best means of ascertaining whether the poor binding affinity to tumours stems from complete *in vivo* deiodination, from loss of biological activity of the radioiodinated EPO, or if the tumoral cell line used is not a good example of an EPOr expressor tumour.

In any event, it seems to be worthwhile to continue our efforts toward developing a new radiopharmaceutical based on EPO. This research work was intended as a first step toward providing new perspectives for the development of this radiotracer. Numerous future studies will be needed to better understand the biological activity of EPO and its process of binding and internalization in the cells of a range of tumours. Acquisition of scintigraphic images of animals injected with ^{123}I-EPO may also be of great importance in evaluating the true capacity of this potential diagnostic radiopharmaceutical.

Given the advances in recent years in the field of molecular imaging, it might also be useful to conduct a study of ^{11}C-labeled EPO as a potential positron emitter radiopharmaceutical. In this case the radioisotope would be a carbon atom, a natural element to the molecule, whose biological activity would remain unchanged after radiolabeling.

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