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Investigation of ^{99m}Tc-labelling of recombinant human interleukin-2 via hydrazinonicotinamide☆

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Abstract

Introduction: Interleukin-2 (IL-2) when radiolabelled with ^{99m}Tc has been proved useful in imaging the side of lymphocytic infiltration in patients with autoimmune disorders and plays a significant role as a T-cell imaging agent. However, the labelling procedures used so far appeared to be rather complex and laborious. The aim of present study was to develop an efficient procedure of ^{99m}Tc-labelling of recombinant human interleukin-2 (rhIL-2) via hydrazinonicotinamide (HYNIC) to develop a dry kit formulation.
Methods: Various molar ratios of rhIL-2/HYNIC (from 1:2 to 1:12) were used at the conjugation step. The conjugates were purified on a PD-10 column to remove the excess of unbound HYNIC, as well as of any aggregates. The final peptide concentration was quantified by the BCA method, and the number of HYNIC molecules incorporated into a rhIL-2 molecule was determined based on the reaction with 2-sulfobenzaldehyde. The ^{99m}Tc-labelling was optimized using various amounts of HYNIC–rhIL-2, ^{99m}Tc, $SnCl_2$, tricine and nicotinic acid (NA). Quality control included GF-HPLC, ITLC, SDS-PAGE and biological assay. Biodistribution studies were performed in Swiss mice and Wistar rats.
Results: Generally, the highest radiolabelling yields were achieved when the HYNIC–rhIL-2 conjugates of ca. 2–4 HYNIC molecule substitution ratios were used. The optimal pH of the reaction medium was found to be in the range of 6.5 to 7.0. GF-HPLC analysis indicated that monomer and aggregates of ^{99m}Tc-HYNIC–rhIL-2 are formed during radiolabelling. At optimized conditions of wet radiolabelling, the ^{99m}Tc-HYNIC–rhIL-2 monomer was obtained with radiochemical purity >99%, specific activity of ca. 4 GBq/mg rhIL-2 and overall yield of ca. 65%. The two-vial freeze-dried kit was prepared: the first vial contained 30 μg HYNIC–rhIL-2, co-ligands, buffer and antioxidant; the second vial contained tricine and $SnCl_2$. The monomer of ^{99m}Tc-HYNIC–rhIL-2 was obtained by gel chromatography on a PD-10 column. No differences between labelled and unlabelled IL2 in terms of biological activity were observed.
Conclusions: Our study shows that rhIL-2 can be efficiently radiolabelled with ^{99m}Tc via HYNIC, with tricine and NA as co-ligands using a two-vial freeze-dried kit. This enables the preparation of sterile and ready-to-use ^{99m}Tc-HYNIC(tricine,NA)-rhIL-2 within 1 h.

Keywords: rhIL-2 peptide; HYNIC; Radiolabelling; Technetium-99m; Inflammation imaging; Biodistribution

1. Introduction

Selected infectious and inflammatory foci can be visualized accurately with radiolabelled cytokines, which act through interaction with specific cell-surface receptors expressed on known cell populations [1,2]. During the past few years, several authors have described the use of cytokines, such as IL-1, which showed specific uptake at the side of infection caused by focal *Staphylococcus aureus* accumulation [3,4]; IL-6 for targeting of acute inflammation [5]; IL-8 for imaging infectious foci, sterile inflammation and osteomyelitis in an animal model and in humans [6–8]; and IL-2 for targeting T lymphocytes and monocytes in chronic, mononuclear cell-mediated

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inflammatory processes such as autoimmune diseases [9–13], kidney graft rejection [14] and lymphocitic infiltration in melanoma characterized by overexpression of CD25 [15].

Chronic inflammation has been successfully targeted by radiolabelled IL-2 by means of specific binding to IL-2 receptors, expressed on activated lymphocytes. It was previously shown by Signore et al. [16] that lymphocytic infiltration in the pancreas could be visualized with ^{123}I-labelled IL-2 within 1 h after injection. Studies in patients with autoimmune disorders such as Hashimoto thyroiditis, Graves' disease, Crohn's disease and celiac disease demonstrated localization of ^{123}I- or ^{99m}Tc-labelled IL-2 at the side of lymphocytic infiltration [17–21].

Therefore, radiolabelled IL-2 is the best available agent for in vivo targeting of mononuclear cell infiltration as present in autoimmune diseases. However, the availability of ^{123}I- or ^{99m}Tc-labelled IL-2 is limited and their preparation is laborious. For clinical application, a simple and rapid labelling procedure of recombinant human interleukin-2 (rhIL-2), using technetium-99m, is preferable. Chianelli et al. [20] reported a two-step synthesis of ^{99m}Tc-IL-2 using the bifunctional chelating agent *S*-tetrahydrofurfurylacetyl-(thio-2,3,5,6-tetrafluorophenyl)-adipylglycylglycine, a ligand with two active sites: one providing a N,S set of donor atoms for coordination of ^{99m}Tc, and the other, a tetrafluorophenyl-active ester, for protein conjugation via the amino groups on lysine residues of IL-2.

An alternative to this method, for ^{99m}Tc-labelling of rhIL-2 using hydrazinonicotinamide (HYNIC) [22,23] as a bifunctional chelator, was recently reported. The HYNIC group is of particular interest because it can be easily labelled with high efficiency (rapid and high-yield radiolabelling). The possibility of developing a dry kit formulation for a convenient preparation of ^{99m}Tc-HYNIC–rhIL-2 was therefore examined.

2. Materials and methods

2.1. Chemicals

Recombinant human IL-2 (Proleukin) was purchased from Chiron Corporation. It is a highly purified protein with a molecular weight of approximately 15,300 Da. The recombinant form differs from native IL-2 in the following manner: rhIL-2 is not glycosylated, it does not have N-terminal alanine, it has serine substituted for cysteine at amino acid position 125 and the aggregation state of rhIL-2 is likely to be different from that of native IL-2. One vial of Proleukin contains 18 million IU (1.2 mg) rhIL-2, 50 mg mannitol and 0.18 mg sodium dodecyl sulfate (SDS), buffered with 0.17 mg monobasic and 0.89 mg dibasic sodium phosphate to pH of ca. 7.2–7.8. Recombinant human IL-2 was produced by recombinant DNA technology using a genetically engineered *E. coli* strain containing an analog of the human IL-2 gene.

N-Hydroxysuccinimidyl hydrazine nicotinate hydrochloride (HYNIC-HCl) was synthesized according to the procedure described by Abrams et al. [24].

Technetium-99m in the form of eluate of sodium pertechnetate from the ^{99}Mo/^{99m}Tc generator MTcG-4 (IAE Radioisotope Centre POLATOM, Świerk, Poland) was used for the study.

All other chemicals and materials were used as supplied and were of analytical or HPLC grade unless otherwise stated.

2.2. Preparation of HYNIC–rhIL-2 conjugate

The procedure of HYNIC conjugation to rhIL-2 was adapted from the procedure described by Rennen et al. [22] and was performed for different molar ratios of HYNIC to rhIL-2 (from 2:1 to 12:1). HYNIC-HCl was dissolved in DMSO at a concentration of 30 mM and was added dropwise to the solution of 600 μg rhIL-2 (2 mg/ml). At the beginning of the conjugation reaction, the pH of the mixture was adjusted to 8.0–8.5 by addition of 1 M $NaHCO_3$. The reaction mixture was incubated for 15 min at room temperature, protected from light. An excess of unbound HYNIC-HCl was removed by purification on a Sephadex G-25 column (PD-10; GE) eluted by 0.05 M phosphate buffer (pH 7.2) containing 0.05% SDS (addition of SDS to the elution buffer increases the solubility of rhIL-2 and prevents aggregation of the protein [25]) and 0.9% NaCl. The HYNIC–rhIL-2 conjugate was collected in the third and fourth 0.5-ml fractions after the void volume.

Determination of the number of HYNIC molecules incorporated into a rhIL-2 molecule was done by convertion of the hydrazino groups to the corresponding hydrazone in reaction with 2-sulphobenzaldehyde (Sigma) which resulted in detected increase absorbance at 343 nm [26]. The protein concentration in the collected fractions was quantified colorimetrically using a bicinchoninic acid-containing reagent (BCA Protein Assay Reagent Kit, Pierce).

2.3. Investigations of ^{99m}Tc-labelling of HYNIC–rhIL-2 conjugates

In the investigation of the ^{99m}Tc labelling conditions of HYNIC–rhIL-2 conjugates, several parameters were evaluated: amounts of HYNIC–rhIL-2 conjugate (from 10 to 60 μg), $^{99m}TcO_4$ (40–1200 MBq), $SnCl_2$ (15–50 μg) and tricine (0.1-50 mg), and different buffer media (pH range from 5.5 to 7.4) were applied for the "wet" radiolabelling studies. In addition, the influence of temperature (from 20°C to 75°C) and duration of radiolabelling (5–120 min) were tested. To prevent aggregation of protein, various amounts of anticoagulants, like SDS or pluronic acid, were also investigated. In further study, nicotinic acid (NA) as additional co-ligand [27,28] was used to improve the stability of the radiolabelled rhIL-2.

All radioactive preparations were analyzed by ITLC, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), GF-HPLC and solid-phase extraction tech-