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In-vitro human leukocyte labeling with ^{64}Cu : An intraindividual comparison with ^{111}In -oxine and ^{18}F -FDG

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Abstract

We investigated labeling human leukocytes (WBC's) in vitro with copper-64 (Cu) comparing labeling efficiency, viability and stability of Cu-WBC's with ^{111}In -oxine (In) WBC's and ^{18}F -FDG (FDG) WBC's.

Methods—Leukocytes from ten volunteers were labeled with Cu, In, and FDG. Forty mL venous blood was collected and leukocyte separation was performed according to standard methods. In-WBC's and FDG-WBC's were labeled according to published methods. For Cu-WBC's, tropolone initially was used as a single chelating agent. Because of poor intracellular Cu retention ($54 \pm 4\%$ at 3 hours, $24 \pm 5\%$ at 24 hours), the fluorinated, membrane-permeable divalent chelator quin-MF/AM was added. WBC's were incubated in 5 mL saline containing 100 μL of 1mM quin-MF/AM in 2% dimethyl sulfoxide and 74-185 MBq Cu-tropolone for 45 minutes at 37°C . Labeling efficiencies, in-vitro cellular viabilities at 1, 3 and 24 hours, and in-vitro stabilities at 1,2,3,4 and 24 hours (except FDG-WBC's), were determined.

Results—Mean Cu-WBC's ($87 \pm 4\%$) and In-WBC's ($86 \pm 4\%$) labeling efficiencies were comparable and were significantly higher than FDG-WBC's ($60 \pm 19\%$, $p < 0.001$). Cell viabilities, similar at 1 hour, were significantly higher for ^{64}Cu -WBC's at three and twenty-four hours. Intracellular retention of activity always was significantly higher for In-WBC's than Cu-WBC's and FDG-WBC's. At 24 hours, intracellular retention was $88 \pm 4\%$, for In-WBC's and $79 \pm 6\%$ for Cu-WBC's.

Conclusion—Cu-WBC's labeling efficiency and viability were comparable or superior to In-WBC's and significantly higher than FDG-WBC's. Although significantly more activity eluted from Cu-WBC's than from In-WBC's, Cu-WBC probably is adequate for imaging. These data suggest that further investigation of in vitro copper-64 labeled leukocytes for PET imaging of infection is warranted.

Keywords

^{64}Cu ; ^{18}F -FDG; ^{111}In -oxine; infection imaging; labeled leukocytes; PET

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Introduction

In vitro labeled leukocyte imaging, using white blood cells (WBC's) labeled either with ^{111}In -oxine (In) or $^{99\text{m}}\text{Tc}$ -exametazime (Tc) is the radionuclide gold standard for evaluating infection and inflammation in the immunocompetent population [1,2]. Positron emission tomography (PET) potentially has several important advantages over conventional gamma camera imaging using single photon emitting agents and several studies suggest that ^{18}F -fluorodeoxyglucose (FDG) is useful for infection and inflammation imaging [3-6]. Although FDG is exquisitely sensitive, it is not specific and accumulates in a variety of conditions, including malignant and benign neoplasms, and fractures, as well as infection and inflammation. The avidity of inflammatory cells for FDG has led to efforts at labeling leukocytes with FDG in vitro, in an attempt to combine the advantages of the labeled leukocyte study with those of PET [7-14]. Although initial results obtained with FDG-WBC's were encouraging, there are significant disadvantages to the procedure. The labeling efficiency of FDG-WBC's is more variable and generally lower than that of In-WBC's [11,12,14]. Another disadvantage is label stability. Pellegrino et al. [13] reported that, in rats, more than 50% of the FDG eluted from leukocytes at 90 minutes and about 80% eluted by six hours. The short physical half-life of fluorine-18 poses logistical challenges such as coordinating delivery of ^{18}F -FDG with patient arrival. It also precludes next day imaging. It is unlikely, therefore, that infection imaging with FDG-WBC's ever will be clinically practical [2].

The ideal PET tracer for labeling leukocytes should have a consistently high labeling efficiency while preserving cell viability. The radiolabel should be stable, with as little elution of radioactivity from the cells as possible, and the physical half life of the radionuclide should be long enough to make in vitro labeling practical and to permit delayed imaging.

Copper-64 (Cu) is an intermediate half-lived positron-emitting radionuclide ($T_{1/2} = 12.7$ hours, e.c. 45%, β^- 37.1%, β^+ 17.9%) suitable for positron emission tomography. The chemistry and in vivo behavior of Cu are well understood, and it has been used both for imaging and therapy [15,16]. The purpose of this investigation was to establish methodology to label WBC's in vitro with Cu and to compare the labeling efficiency, viability, and stability of Cu-WBC's with those of ^{111}In -WBC's and FDG-WBC's.

Materials and Methods

Leukocyte Separation

Ten healthy volunteers, seven men and three women, 30 years to 57 years of age, were enrolled in this investigation. *Leukocyte separation was performed in a manner similar to previously published methods (17, 18).* For all labelings, 40 mL of venous blood were collected in a syringe anticoagulated with heparin. Seven mL of a settling agent, hydroxyethyl starch (6% hetastarch in 0.9% NaCl solution), were added to the syringe, which was maintained in a vertical position in a laminar flow hood and allowed to gravity sediment at room temperature for 60 to 90 minutes. A 21-gauge butterfly then was attached to the syringe, and the leukocyte/platelet rich plasma supernatant was collected slowly into two conical 14 mL plastic tubes and then centrifuged at 450 G for ten minutes. The platelet poor plasma (PPP) supernatant was removed and used for the final suspension of leukocytes, leaving the WBC pellet at the bottom of the tube. The pellet was resuspended in 5 mL sterile saline containing heparin and the suspension was centrifuged again for ten minutes. The supernatant was discarded and the WBC pellet was resuspended in 5 mL normal saline and labeled with In, FDG, or Cu.

Radiolabeling of Leukocytes

In-WBC and FDG-WBC's were labeled according to published methods [8,19]. Cu is a positively charged metal and in order for it to cross the cell membrane a neutral complex must be formed. We initially investigated the feasibility, in three subjects, of using tropolone (T) as the chelating agent for Cu-WBC's. The Cu-T complex was prepared by adding 74-185 MBq Cu to 50 µg of tropolone in 1 mL HEPES buffer, pH 7.6. The mixture was maintained at room temperature for ten minutes. The leukocyte pellet was incubated with the Cu-T complex at 37° C for 30 minutes. The Cu-T-WBC's then were isolated by centrifugation, and resuspended in 5 mL of PPP.

The labeling procedure subsequently was modified by adding a second chelating agent, the fluorinated, membrane-permeable divalent chelator quin-MF/AM [2-(2-amino-4-methyl-5-fluorophenoxy) methyl-8-aminoquinoline-N, N, N', N'-tetra-acetic acid]. *The leukocyte pellet was incubated in 5 mL saline with 100 µL of 1mM quin-MF/AM in dimethyl sulfoxide (2% concentration) and 74-185 MBq ⁶⁴Cu-T for 45 minutes at 37°C. At the end of incubation, 5 mL PPP was added and the mixture was centrifuged again at 450 G for 5 minutes. The supernatant was removed and the pellet resuspended in 5 mL PPP. The mixture again was centrifuged at 450 G for 5 minutes. To eliminate any residual dimethyl sulfoxide and extracellular Quin-MF/AM, the supernatant was removed and the Cu-WBC's were resuspended in 5 mL fresh PPP.*

Labeling efficiencies (LE) were calculated using the formula $LE = C/(C + W) \times 100$, where C was the cell associated activity and W was the activity associated with the supernatant. In vitro leukocyte viability was assessed using the trypan dye exclusion technique [20]. Microscopic examination was performed on aliquots of labeled leukocytes at 1 hour, 3 hours and 24 hours after labeling. Cell viability (V) was calculated using the formula $V = N/(N + B) \times 100$, where N represented the number of viable cells and B the number of trypan dye containing cells. A minimum of one hundred cells were counted for all viability measurements. To determine the in vitro stability of the various preparations, radiolabeled WBC's were washed with PPP to remove unincorporated or loosely bound label, then resuspended in 5 mL PPP and maintained at 37° C. At 1, 2, 3, 4 and 24 (except FDG-WBC's) hours after labeling, 1 mL samples were withdrawn, diluted with 4 mL saline and centrifuged at 450 G for 5 minutes to pellet the cells. Activity in the cell pellet and supernatant were measured separately to determine the amount of activity released from the labeled leukocytes. Intracellular retention of radioactivity was calculated using the formula: $Retention (R) = AC/(AC + AS) \times 100$, where AC was the cellular pellet activity and AS was the activity in the supernatant.

Statistics

Statistical analyses were performed using commercially available software (Medcalc, Version 7.5.0.0., Medcalc Software, Inc., Mariakerke, Belgium). Values are reported as means ± one standard deviation. Frequencies and percentages were used to characterize categorical variables. The D'Agostino-Pearson test was used to determine whether continuous variables were normally distributed. One-way analysis of variance (ANOVA) was used to determine whether differences were significant among the labeling methods, and when they were, paired t-tests were used to further test the hypothesis that mean values were not different between methods. Correlation between continuous variables was determined using linear regression, which generated Pearson's correlation coefficients. Bland-Altman analyses of measurement differences plotted versus mean values were used to assess biases, trends and limits of agreement. Mean values were compared using a Bonferroni corrected two-tailed paired-T-test with a value of $p < 0.05/(v-1)$ for v degrees of freedom as significant.

Results

The mean labeling efficiency for Cu-T-WBC's (n=3) was $83 \pm 4\%$. At three hours $54 \pm 4\%$ of the radioactivity was retained intracellularly. At 24 hours, $24 \pm 5\%$ of the radioactivity was retained intracellularly (Figure 1). Because of the amount of Cu elution from the cells, Cu-T-WBC's were not investigated further.

Labeling efficiency, cell viability and label stability were normally distributed for Cu-WBC's, In-WBC's, and FDG-WBC's, for all time points ($p > 0.05$ in each case). Labeling efficiencies were significantly different by ANOVA (F-ratio = 16.4, $p < 0.001$). The mean labeling efficiencies of Cu-WBC's ($87 \pm 4\%$) and In-WBC's ($86 \pm 4\%$) were comparable ($p = 0.62$), and were significantly higher than that of FDG-WBC's ($60 \pm 19\%$) ($p = 0.005$) (Table 1).

In vitro cell viabilities for all three labeling methods were similar at 1 hour ($p = 0.93$), but viability of Cu-WBC's was significantly higher than those of In-WBC's and FDG-WBC's at three hours ($p = 0.02$) and at 24 hours ($p < 0.001$) (Table 2).

The stabilities of the three labels were significantly different at each time point ($p < 0.001$). At all time points, there was significantly more retention of the radiolabel in In-WBC's than in Cu-WBC's and FDG-WBC's ($p < 0.001$). At all time points radiolabel retention in Cu-WBC's was significantly more than that in FDG-WBC's (Table 3).

Discussion

Positron emission tomography (PET) offers important advantages over conventional gamma camera imaging using single photon emitting agents and the role of FDG-PET for imaging infection and inflammation is increasing. Although it is exquisitely sensitive, FDG is not specific and accumulates in a variety of conditions in addition to infection and inflammation. The avidity of inflammatory cells for FDG has led to attempts at labeling leukocytes with FDG in vitro and initial results obtained with FDG-WBC's were encouraging [10-13]. There are, however, significant disadvantages to the procedure and it is unlikely that FDG-WBC imaging ever will be clinically practical [2].

The ideal PET tracer for labeling leukocytes should have a consistently high labeling efficiency while preserving cell viability. The physical half life of the tracer should be long enough to make in vitro labeling practical and to permit imaging up to 24 hours after reinjection of labeled cells. The radiolabeled complex should be stable, with as little elution from the leukocytes as possible.

Copper-64 is an intermediate half-lived positron-emitting radionuclide ($T_{1/2} = 12.7$ hour, e.c. 45%, β^- 37.1%, β^+ 17.9%). Its chemistry and in vivo behavior are well understood, and it has been used both for imaging and therapy [15,16]. Cu, like In, is a positively charged metal, and requires the use of a chelating agent in order to enter the leukocyte. Our initial approach to Cu-WBC labeling, therefore, was to use methodology similar to that which has been used for labeling leukocytes with In. We used tropolone because it is a water soluble compound that chelates ionic Cu and, unlike oxine, does not have to be extracted with chloroform [21,22]. Although the labeling efficiency for Cu-T-WBC's was satisfactory ($83 \pm 4\%$), the complex was not stable, with nearly 50% of the activity released from the cells by three hours and more than 70% released by 24 hours. The explanation for the instability is uncertain; there may be a lack of proteins within the leukocyte to which the ^{64}Cu could bind.

Regardless of the explanation, it was necessary to modify the procedure and a second chelating agent, the fluorinated, membrane-permeable, calcium chelator quin-MF/AM was added. This agent, which is used to measure intracellular free calcium by ^{19}F NMR, crosses the leukocyte

cell membrane in its acetoxymethyl ester form, but does not complex with Cu. Once inside the cell, however it is hydrolysed by intracellular esterases to the negatively charged anionic form which has a very high affinity for Cu^{2+} (dissociation constant $< 50 \text{ nM}$). The hydrolysed form of the compound rapidly chelates Cu from the Cu-T complex, and the Cu-Quin/MF complex is trapped within the cell [23].

The labeling efficiency, using the dual chelator technique, of Cu-WBC's which was consistent among the ten subjects studied, was nearly identical to that of In-WBC's, and was significantly higher and less variable than that of FDG-WBC's. The mean labeling efficiency for Cu-WBC's in this investigation also was higher than the 50%-55% labeling efficiency that has been reported for non-stabilized Tc-WBC's, and similar to what has been reported for stabilized Tc-WBC's [24,25].

Successful labeled leukocyte imaging requires the presence of viable cells. In vitro viability of Cu-WBC's was comparable to those of In-WBC's and FDG-WBC's at 1 hour. At 3 and 24 hours viability of Cu-WBC's was significantly higher.

Another important requisite for successful leukocyte imaging is label stability, in order to assure that foci of activity reflect accumulation of labeled leukocytes. Although significantly more activity was released from Cu-WBC's than from In-WBC's at all time points, the differences in the percent elution at each time point were relatively small (Table 3). The 11% to 14% percent elution from Cu-WBC's at 2-4 hours, moreover, is similar to the 12%-14% elution that has been reported for unstabilized Tc-WBC's at 1 to 2 hours (24). Consequently, this degree of elution should not, we believe, adversely affect Cu-WBC imaging.

Conclusions

This investigation demonstrates the feasibility of labeling human leukocytes with the positron emitter ^{64}Cu , which in vitro, is superior to FDG labeled leukocytes. These results suggest that the potential of copper-64 labeled leukocytes for imaging infection warrants further investigation.

Acknowledgments

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Figure 1. Percent retention versus time for blood samples of three individuals for tropolone alone. When using tropolone as the chelating agent, there was rapid elution of Cu from leukocytes with nearly 50% of the activity eluted by 4 hours and more than 70% eluted by 24 hours.

Table 1

Labeling Efficiencies

Radionuclide	Mean Labeling Efficiency	Labeling Efficiency Range
Cu-WBC	87±4%	80% - 93%
In-WBC	86±4%	81% - 93%
FDG-WBC	60±19% *	28% - 99%

* p < 0.05 versus ⁶⁴Cu-WBC, ¹¹¹In-WBC

Table 2

Cell Viability

Viability			
Radionuclide	1 hour	3 hour	24 hour
⁶⁴ Cu-WBC	99±1%	98±1%	61±8%
¹¹¹ In-WBC	99±1%	96±2% *	48±5% *
¹⁸ F-FDG-WBC	99±1%	96±2% *	50±7% *

*
p < 0.05 versus ⁶⁴Cu-WBC

Table 3

Intracellular Radiolabel Retention

Radionuclide	Retention (%)				
	1 hour	2 hours	3 hours	4 hours	24 hours
⁶⁴ Cu-WBC	91±4% [§]	89±4% [§]	88±4% [§]	86±4% [§]	79±6%
¹¹¹ In-WBC	94±2% [*]	93±3% [*]	92±3% [*]	91±3% [*]	88±4% [*]
¹⁸ F-FDG-WBC	85±4%	81±4%	76±4%	68±7%	—

^{*} p < 0.05 for ¹¹¹In-WBC higher than ⁶⁴Cu-WBC & ¹⁸F-FDG-WBC

[§] p < 0.05 for ⁶⁴Cu-WBC higher than ¹⁸F-FDG-WBC