Journal of Labelled Compounds and Radiopharmaceuticals
J Label Compd Radiopharm 2007; **50**: 211–217.
Published online in Wiley InterScience
(www.interscience.wiley.com). DOI: 10.1002/jlcr.1207

JLCR

Research Article

Preparation and *in vitro* evaluation of ^{99m}Tc-labelled bovine lactadherin as a novel radioligand for apoptosis detection

LASSE N. WAEHRENS[1], JAN T. RASMUSSEN[1], CHRISTIAN W. HEEGAARD[1] and LISE FALBORG[2,*]

[1] Protein Chemistry Laboratory, Department of Molecular Biology, University of Åarhus, Denmark
[2] Department of Nuclear Medicine, Åarhus University Hospital, Denmark

Received 1 November 2006; Revised 24 November 2006; Accepted 25 November 2006

Abstract: Lactadherin is an opsonin, which binds with high affinity to phosphatidylserine exposed on the surface of apoptotic cells via its C1C2 domains. Phagocytosis of the apoptotic cells is then facilitated by an Arg-Gly-Asp (RGD)-mediated binding to macrophages surface integrins. The present report describes the synthesis of ^{99m}Tc-HYNIC–lactadherin with a radiochemical yield of $88 \pm 5\%$ ($n = 3$) and a specific activity of $41 \pm 5\,\mu$Ci/μg ($n = 3$) when purified. Purified ^{99m}Tc-HYNIC–lactadherin was shown to be stable for at least 5 h when supplemented with 1.5 mg/ml fatty-acid-free BSA. The radiolabelled protein retained its phospholipid binding ability that was verified by its ability to bind to apoptotic HL60 leukaemia cells. The apoptotic cells demonstrated a RGD independent 3- to 4-fold excess binding of the radioactive component relative to control cells. Surplus of unlabelled lactadherin almost completely inhibited the binding of ^{99m}Tc-HYNIC–lactadherin. Collectively our data indicate that ^{99m}Tc-HYNIC–lactadherin is potentially useful as a new molecular binding tool for the identification of apoptotic cells.

Keywords: lactadherin; apoptotic imaging; *in vitro*; HYNIC; phosphatidylserine

Introduction

Externalization of the negatively charged phosphatidylserine (PS) molecules in the plasma membrane has become an early hallmark feature utilized in apoptosis imaging today. PS exposure is crucial for recognition and phagocytosis.[1]

It has previously been shown that the structurally conserved annexin protein family is capable of binding to negatively charged phospholipids in a Ca^{2+}-dependent way.[2] Thus, several members of the annexin family of proteins, most notably hereof Annexin V,[3,4] have been used for radioactive imaging of apoptosis utilizing their ability to bind to PS.[5–7]

The protein lactadherin (also known as PAS-6/7 or MFG-E8) has also been shown to bind specifically to PS in phospholipid membranes, however, in a calcium-independent manner.[8–10] Lactadherin (bovine) exists as two glycosylation variants of the same protein core (45.6 KDa) and the apparent masses on SDS-PAGE are 47 and 52 kDa.[11] Lactadherin comprises a C-terminally located phospholipid binding C1C2 domain and two N-terminally EGF-like domains containing one RGD sequence that is able to recognize $\alpha_V\beta_5$ and $\alpha_V\beta_3$ integrins.[8] The dual binding activity of lactadherin has been shown to be an important structural feature, enabling it to be an important component of the immune system facilitating phagocytosis of apoptotic cells.[12,13] This study demonstrates that lactadherin can be conjugated to HYNIC and subsequently labelled with ^{99m}Tc. Stability studies and *in vitro* binding studies confirm the versatility of this reagent. It is hypothesized that ^{99m}Tc-HYNIC–lactadherin can be used as a Ca^{2+}-independent probe for detection of apoptosis.

Materials

Chemicals

All chemicals (analytical grade) were supplied by Sigma–Aldrich Corp or Merck & Co., Inc, beside the specified exceptions. DMSO extra pure was purchased from Ferak. ^{99m}Tc was from Tyco Healthcare. ACD-buffer was manufactured in house at the pharmacy of

*Correspondence to: Lise Falborg, Department of Nuclear Medicine, Åarhus University Hospital, Denmark. E-mail: lfalb@as.aaa.dk

Aarhus Sygehus. Fatty acid-free bovine serum albumine (BSA), Fraction V was from Calbiochem.

Cell culture

HL60 (human leukaemia) cells were purchased from the European Collection of Cell Cultures and maintained in Iscoves Modified Dulbeccos Media + GlutaMAX ™ media with 20% fetal bovine serum (FBS) and 1% Pen/Strep at 37°C in 5% CO_2. Media, FBS and Pen/Strep were from Invitrogen.

Equipment

TLC strips were ITLC-SG from Pall Lifesciences. The Gamma counter was a Cobra Auto-gamma from Packard. Radiolabelled lactadherin was purified by HPLC (Dionex) using a Biosep SEC-S 3000 column attached to a GFC-3000 precolumn (Phenomenex) and mounted on a HPLC (Dionex). The radiation counter was a 2″ socket 81030043 with a Gabi Star from Raytest. The HPLC software was Chromeleon 6.5 SP3 build 980. Dialysis tubing was from Medicell International Ltd with a cut-off of 12–14 000 daltons. The microscope was a LSM 510 META equipped with a LSM 510 Laser Module and controlled by the LSM 510 3.2 SP2 software from Zeiss.

Methods

Protein purification

Bovine lactadherin was purified as previously described[11] and stored at −80°C in 75 mM sodium phosphate, pH 7.0. Protein concentrations were determined by amino acid analysis (OPA-based) or ELISA performed as reported in earlier studies.[14] Purity was checked by SDS-PAGE and amino acid sequencing.

Preparation of ^{99m}Tc-HYNIC–lactadherin and radiochemical analysis

Succinimidyl-hydrazinonicotinamide (S-HYNIC) was synthesized as previously described. [15]HYNIC-conjugated lactadherin was made by a modification of the methods used on annexin V[16] and IgG.[15]

S-HYNIC dissolved in dimethylformamide (DMF) (41.5 mM) was added to lactadherin (0.66 mg/ml) in 75 mM Na_2HPO_4, pH 7.0, with a molar ratio of 10:1 of HYNIC and lactadherin. Following 3 h incubation, in the dark at room temperature under continuous gentle agitation, the reaction was stopped by adding 0.2 ml 0.5 M Glycine in PBS, pH 7.4, and left for additional 10 min. The sample was then dialyzed against 20 mM tri-sodiumcitrate, 100 mM NaCl, at 5°C, in successive changes of decreasing pH (7.4–5.2). The conjugated protein sample was stored at –80°C in aliquots of 100 µl.

Incorporation of HYNIC into lactadherin was evaluated.[17] Briefly, 0.9 ml of 50 mM sodium acetate, pH 4.73, 100 mM NaCl, 2.5% acetonitrile (vol/vol) were mixed with 100 µl HYNIC–lactadherin in 20 mM tri-sodiumcitrate, 100 mM NaCl, pH 5.2 and 6.6 µl 4-nitrobenzaldehyde (10 mg/ml in DMF). This was allowed to react for 4.5 h in the dark at room temperature under continuous, gentle agitation. During the incubation, 4-nitrobenzaldehyde converts the hydrazine group of HYNIC to its corresponding hydrazone, which absorbs at 385 nm (ε=25 300 $M^{-1}\,cm^{-1}$). Lactadherin concentration was determined by measuring at A_{280} (ε=77 180 $M^{-1}\,cm^{-1}$, calculated).

To couple HYNIC–lactadherin with ^{99m}Tc, 5 µl of $SnCl_2$ (5 mg/ml in 0.05 M HCl) and 10 µl tricine (100 mg/ml in 20 mM tri-sodiumcitrate, 100 mM NaCl, pH 5.2) were mixed. HYNIC–lactadherin (100 µl) was added, mixed and followed by 100 MBq of $^{99m}TcO_4^-$. The mixture was incubated for 15 min in the dark at room temperature under continuous agitation.

The conjugate was separated on a Biosep-SEC-S 3000 HPLC column equilibrated in PBS, pH 7.4 (flow 1 ml/min). A fraction eluting from 16.5 to 19.5 min was collected and used. Fatty acid-free BSA was added to reach a concentration of 1.5 mg/ml. Before purification the radiochemical purity was determined by TLC in 100% acetone and in 68 mM citrate, pH 5.0, 74 mM dextrose (ACD buffer).[18]

In vitro stability

Aliquots of freshly prepared ^{99m}Tc-HYNIC–lactadherin, with and without 1.5 mg/ml fatty acid-free BSA, were subjected to TLC in ACD buffer and acetone once every hour over a 5 h period to determine the radiochemical purity (RCP). During this period the ^{99m}Tc-HYNIC–lactadherin preparations were incubated at room temperature protected from light. The stability with and without BSA carrier was calculated by cutting the TLC strips and measuring the activity in a gamma counter.

In vitro binding assay

HL60 cells (Human Leukaemia cell line) were split into concentrations of 10^6 cells/ml and incubated for 24 h in 6 well plates with 10 µM etoposide dissolved in DMSO.[19] The cells were harvested by centrifugation (450*g*) and washed once with Tyrodes buffer (15 mM HEPES, 3.3 mM NaH_2PO_4, pH 7.4, 138 mM NaCl,

DOI: 10.1002.jlcr

2.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM dextrose, and 0.2% fatty acid-free BSA). The cells were then resuspended in Tyrodes buffer at a concentration of 10^6 cells/ml and aliquots (0.25 ml) were placed in 1.5 ml micro-centrifuge tubes. Multiple cell aliquots were added to different amounts of radioactive ^{99m}Tc-HYNIC–lactadherin for 15 min in the dark at room temperature. All ^{99m}Tc-HYNIC–lactadherin was prestabilized with 1.5 mg/ml fatty acid-free BSA and the experiments were done in triplicate. To each sample, Tyrodes buffer was added for a total volume of 1 ml followed by 5 min of centrifugation (14 000*g*). The supernatant was removed and stored in a counting tube. The pellet was washed once in 1 ml Tyrodes buffer, followed by resuspension in 0.5 ml Tyrodes buffer and transferred to a counting tube. The counts were collected and the percentage of cell bound radioactivity was assessed. The procedure was repeated with untreated HL60 cells to evaluate ^{99m}Tc-HYNIC–lactadherin binding to non-apoptotic cells. Statistical analysis on the collected data was done utilizing the Student's *t*-test.

In vitro protein domain specificity assay

The experiment was done using the same set-up as with the binding assay, except that the samples were added none of the three mixtures: 100 µl ^{99m}Tc-HYNIC–lactadherin, ^{99m}Tc-HYNIC–lactadherin supplemented with 100-fold excess of unlabelled lactadherin, ^{99m}Tc-HYNIC–lactadherin supplemented with a 932-fold excess of RGD, respectively. Triplicate samples were prepared for each variation.

Results

HYNIC conjugation and radiochemical purity

After conversion of the intrinsic hydrazine group to hydrazone, the amount of conjugated HYNIC per protein molecule was determined spectrophotometrically to be 4. A radiochemical purity analysis performed by TLC prior to purification revealed no $^{99m}TcO_4^-$ at the solvent front (acetone). By developing the TLC strips in ACD buffer the RCP could be determined to be $88 \pm 5\%$ ($n = 3$). Impurities can be interpreted as ^{99m}Tc-tricine and colloidal ^{99m}Tc. The specific activity of the purified product was established to be 1.5 ± 0.2 MBq/µg lactadherin (41 ± 5 µCi/µg) ($n = 3$) at the end of production. The procedure of labelling HYNIC-conjugated lactadherin with ^{99m}Tc is relatively fast with an overall production time of 35–40 min.

In vitro stability

The radioactively labelled lactadherin was separated from excess $SnCl_2$ and tricine by the use of a size-exclusion HPLC purification process. A representative chromatogram is given in Figure 1. The protein eluting between 16.5 and 18.5 min was collected and divided into two fractions, and fatty acid-free BSA solution (final conc. 1.5 mg/ml) was added to one of the fractions. The relatively low yield of the HPLC purification ($19 \pm 2\%$, *n*=3, measured as the recovered relative to the injected activity) relates to the fact that collection was restricted to the area with the highest absorbance, and that lactadherin most likely adhered to the

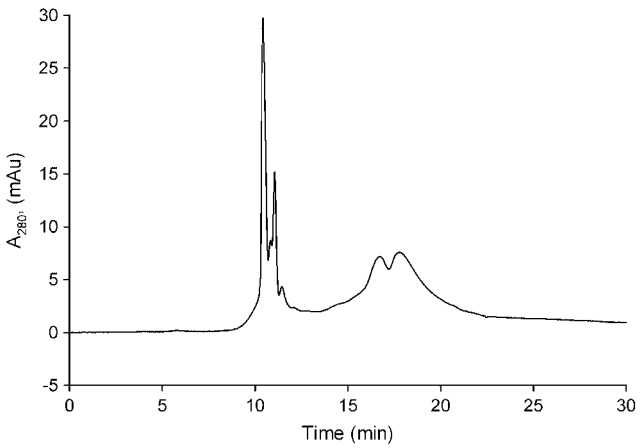

Figure 1 A typical size-exclusion HPLC chromatogram of ^{99m}Tc-HYNIC–lactadherin. One hundred/µl HYNIC–lactadherin was mixed with 5 µl of SnCl2 (5 mg/µl in 0.05 M HCl), 10 ml tricine (100 mg/ml in 20 mM tri-sodiumcitrate, 100 mM NaCl, pH 5.2) and 100 MBq ^{99m}TcO4- for 15 min in the dark at room temperature with continuous agitation. The mixture was injected onto a Biosep-SEC-S 3000 HPLC column equilibrated in PBS, pH 7.4. Absorbance is shown at 280 nm.

DOI: 10.1002.jlcr

column. The overlapping double peak may indicate that the two glycosylation variants of lactadherin are separated to some degree on this column.

The results of the successive TLC stability tests are shown in Figure 2. As one might have expected, BSA stabilized the protein solution considerably. Accordingly, in the presence of BSA the RCP only decreases from 94 to 92% during a 5-h period. The non-stabilized solution plunges from 90 to 35% RCP during the same period. It was therefore decided that the binding experiments addressing the biological function should all be performed with the BSA stabilized ^{99m}Tc-HYNIC–lactadherin solutions and finished before 5 h after the radioactive labelling.

In vitro binding assay

An *in vitro* binding assay was established in order to determine if lactadherin were still biologically active after conjugation with HYNIC and labelling with ^{99m}Tc. HL60 cells were primed for apoptosis, and thereby PS exposure, by addition of etoposide (10 μM) for a 24 h incubation period. The appearance of an apoptotic phenotype was documented by monitoring the etoposide treated and untreated cells microscopically (Figure 3). Binding of ^{99m}Tc-HYNIC–lactadherin to cells was determined by adding increasing amounts of radioactive lactadherin to the destined cell samples, followed by sedimentation of the cells. By comparing the radioactivity in the pellet (bound) and the supernatant (unbound), a concentration-dependent progressive binding of lactadherin was found for the etoposide treated cells (Figure 4). Significant difference in binding activity between apoptotic and control cells (136 ± 6.6%) was achieved with a ^{99m}Tc-HYNIC–lactadherin concentration as low as 11.6 nM. Furthermore, the difference in binding to apoptotic and normal HL60 cells reached a plateau at 30.9'nM label resulting in an 320 ± 16.9% increase.

In vitro protein domain specificity assay

Experiments were conducted to investigate if binding of radiolabelled lactadherin to the apoptotic cells is

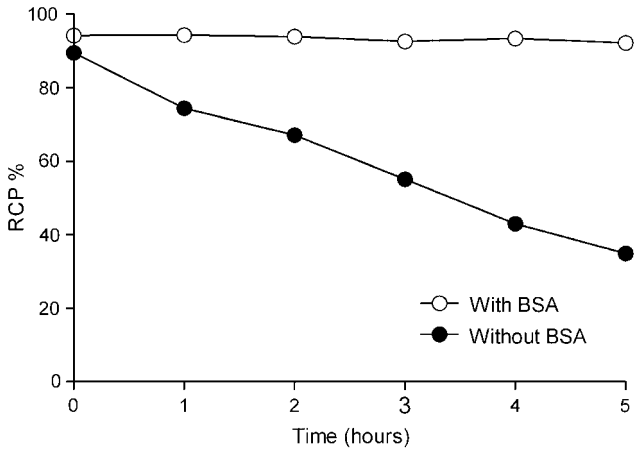

Figure 2 The stability of ^{99m}Tc-HYNIC–lactadherin (1.5 μg/ml) with and without BSA (1.5 mg/ml analysed by thin layer chromatography.

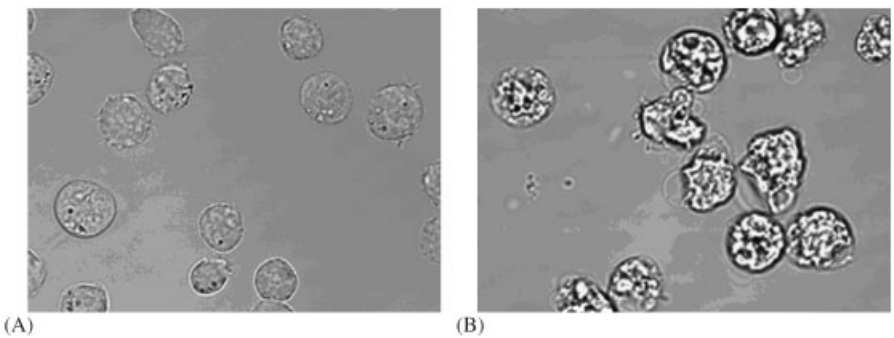

Figure 3 Confocal microscopy images of HL60 cells cultured with or without addition of etoposide: (A) untreated cells and (B) cells incubated for 24 h with 10 μM etoposide. The treated cells display all the classical morphological features of apoptosis.

DOI: 10.1002.jlcr

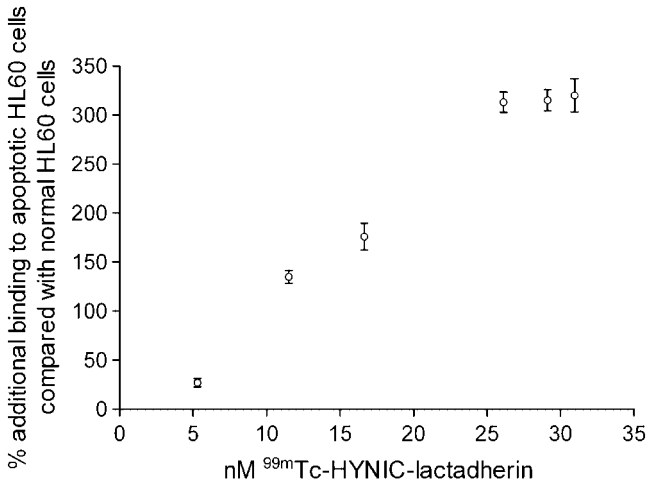

Figure 4 Specific binding of ^{99m}Tc-HYNIC–lactadherin to apoptotic HL60 cells at different ^{99m}Tc-HYNIC–lactadherin concentrations. HL60 cells were incubated with 10 μM etoposide for 24 h. The cells were washed and incubated with ^{99m}Tc-HYNIC–lactadherin for 15 min followed by centrifugation. The percentage of total radioactivity in the pellet of each sample (n=3) was determined and compared with data from control experiments with untreated HL60 cells. The concentration of ^{99m}Tc-HYNIC–lactadherin in each sample was determined by ELISA (n=3).

Table 1 Domain specificity of ^{99m}Tc-HYNIC–lactadherin

Etoposide	Unlabelled lactadherin	RGD	^{99m}Tc-HYNIC–lactadherin	% radioactivity in pellet (n=3)	SD
–	–	–	+	13.1	0.25
–	+	–	+	0.4	0.02
–	–	+	+	13.0	0.19
+	–	–	+	35.7	0.81
+	+	–	+	2.0	0.14
+	–	+	+	35.6	0.63

mediated by the inherit integrin or PS binding sites. This was done by adding excess amounts of the RGD peptide (932 ×) or unlabelled lactadherin (100 ×) together with 19.4 nM ^{99m}Tc-HYNIC–lactadherin to normal and apoptotic HL60 cells, respectively (Table 1). Unfortunately, it was only possible to use lactadherin in a 100-fold excess, due to the limited solubility of the protein. Adding more would thus dilute the sample to a degree that would render the experiment unusable. Without the RGD peptide additional 172 ± 8% radioactivity from ^{99m}Tc-HYNIC–lactadherin was found in the cell fraction of etoposide treated cells compared to untreated cells. By comparison, inclusion of RGD did not dramatically change the difference in cell-associated radioactivity between etoposide treated and untreated cells, as it was determined to be 173 ± 6%. As RGD normally quenche lactadherins integrin binding, the data indicate that the binding to etoposide treated cells takes place independently of integrins. Contrary, when native lactadherin was added in excess, the radioactivity in the pelleted apoptotic cells dropped to 2 ± 0.14%. This strongly suggests that the binding of ^{99m}Tc-HYNIC–lactadherin to apoptotic cells is mediated by the same mechanisms as for unlabelled lactadherin. Interestingly, when surplus amounts of unlabelled lactadherin were added to non-apoptotic cells this effectively inhibited the background binding by ^{99m}Tc-HYNIC–lactadherin, suggesting that untreated HL60 cells also present a minor quantity of lactadherin-specific binding sites.

Discussion

The *in vitro* experiments clearly indicate that ^{99m}Tc-HYNIC–lactadherin is a possible option for evaluation of apoptosis *in vitro*. The conjugation of HYNIC to lactadherin was made with a low protein concentration of 0.66 mg/ml not as a choice, but because purified

DOI: 10.1002.jlcr

lactadherin in isotonic solution has a strong tendency to precipitate above this concentration (data not shown). This, in itself, makes the protein solution dilute compared to reports on annexin V[20] labelling.

The stability studies showed that ^{99m}Tc-labelled lactadherin could be produced with a very small decrease in radiochemical purity over 5 h, when it was stabilized with BSA. The impact of adding BSA was evident at a very early stage. In fact, it looks as if the RCP already decreases a little during the time it took to take an aliquot, apply it to the TLC plate and initiate development. This stresses the importance of using a stabilizing agent as early as possible.

The results showed that ^{99m}Tc-HYNIC–lactadherin retains enough of its affinity for PS to generate a significant leap in binding to cells presenting this lipid on their surface. It is effectively ruled out that this binding is due to RGD-mediated interactions with integrins, because the affinity for the apoptotic cells was unaffected by presence of high amounts of the RGD tripeptide. This strongly indicates that the PS binding C1C2 domain of lactadherin is responsible for the specific interactions, which corresponds well with earlier findings.[8,12] The background level of specific lactadherin binding to non-apoptotic cells is not likely to reflect a constitutive PS exposure by untreated cells, but is most properly due to the mechanical handling of the cells.

Studies have shown that bovine lactadherin, in comparison with annexin V, has the strongest inhibitory effect on PS-induced activation of factor X_a in the blood coagulation cascade.[9] This inhibitory effect is mediated by competition for PS-containing binding sites on the surface of platelets. A further investigation concluded that lactadherin has a higher binding affinity for phospholipid-coated beads in the interval of 0–2% PS in a Ca^{2+}-independent manner.[10] Annexin V shows little affinity for phospholipid-coated beads with less than 4% PS at physiological Ca^{2+} concentrations.[21,22] These differences in PS binding abilities of annexin V and lactadherin, combined with the present findings make lactadherin a very interesting protein for apoptosis imaging *in vitro* and may be also *in vivo*.

It has been conclusively shown that lactadherin is an important part of the immune system. Knockout mice deficient in lactadherin (dubbed MFG-E8 in mice), show that tangible body macrophages present in the germinal centres of the spleen and lymph nodes have impaired uptake of apoptotic cells. While wild-type mice show several condensed apoptotic nuclei inside tangible body macrophages, the knockout mice simply show the macrophages wrapped around the apoptotic bodies, but without phagocytosis.[23] Recent reports reveal that lactadherin detects PS expression earlier than annexin V on thrombin-activated platelets and apoptotic cells of the HL60 and K562 cell lines, thus stressing the potential of lactadherin in imaging.[24,25] Lactadherin, being an innate part of the immune system, is interesting when considering preclinical *in vivo* experiments utilizing the labelling procedure described here.

Conclusion

^{99m}Tc-HYNIC–lactadherin has been synthesized, tested for stability and *in vitro* biological binding activity. The data clearly show that the compound is a potential imaging agent for apoptosis recognition.

REFERENCES

1. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. *J Immunol* 1992; **148**: 2207–2216.
2. Gerke V, Moss SE. *Physiol Rev* 2002; **82**: 331–371.
3. Tait JF, Gibson D, Fujikawa K. *J Biol Chem* 1989; **264**: 7944–7949.
4. Meers P, Mealy T. *Biochemistry* 1993; **32**: 11711–11721.
5. Blankenberg FG, Katsikis PD, Tait JF, Davis RE, Naumovski L, Ohtsuki K, Kopiwoda S, Abrams MJ, Strauss HW. *J Nucl Med* 1999; **40**: 184–191.
6. Zijlstra S, Gunawan J, Burchert W. *Appl Radiat Isot* 2003; **58**: 201–207.
7. Luo Q, Zhang Z, Wang F, Lu H, Guo Y, Zhu R. *Biochem Biophys Res Commun* 2005; **335**: 1102–1106.
8. Andersen MH, Graversen H, Fedosov SN, Petersen TE, Rasmussen JT. *Biochemistry* 2000; **39**: 6200–6206.
9. Shi J, Gilbert GE. *Blood* 2003; **101**: 2628–2636.
10. Shi J, Heegaard CW, Rasmussen JT, Gilbert GE. *Biochim Biophys Acta* 2004; **1667**: 82–90.
11. Hvarregaard J, Andersen MH, Berglund L, Rasmussen JT, Petersen TE. *Eur J Biochem* 1996; **240**: 628–836.
12. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. *Nature* 2002; **417**: 182–187.
13. Asano K, Miwa M, Miwa K, Hanayama R, Nagase H, Nagata S, Tanaka M. *J Exp Med* 2004; **200**: 459–467.
14. Kvistgaard AS, Pallesen LT, Arias CF, Lópes S, Petersen TE, Heegaard CW, Rasmussen JT. *J Dairy Sci* 2004; **87**: 4088–4096.
15. Blankenberg FG, Katsikis PD, Tait JF, Davis RE, Naumovski L, Ohtsuki K, Kopiwoda S, Abrams MJ,

DOI: 10.1002.jlcr

Darkes M, Robbins RC, Maecker HT, Strauss HW. *PNAS* 1998; **95**: 6349–6354.
16. Abrams MJ, Juweid M, tenKate CI. *J Nucl Med* 1990; **31**: 2022–2028.
17. King TP, Zhao SW, Lam T. *Biochemistry* 1986; **25**: 5774–5779.
18. Ercan MT. *Appl Radiat Isot* 1992; **43**: 1175–1177.
19. Liu WM, Joel SP. *Cancer Chemother Pharmacol* 2003; **51**: 291–296.
20. Verbeke K, Kieffer D, Vanderheyden J-L, Reutelingsperger C, Steinmetz N, Green A, Verbruggen A. *Nucl Med Biol* 2003; **30**: 771–778.
21. Andree HAM, Reutelingsperger CPM, Hauptmann R, Hemker HC, Hermens WT, Willems GM. *J Biol Chem* 1990; **265**: 4923–4928.
22. Tait JF, Gibson D. *Arch Biochem Biophys* 1992; **298**: 187–191.
23. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, Nagata S. *Science* 2004; **304**: 1147–1150.
24. Dasgupta SK, Guchait P, Thiagarajan P. *Tranl Res* 2006; **148**: 19–25.
25. Shi J, Shi J, Waehrens LN, Rasmussen JT, Heegaard CW, Gilbert GE. *Cytometry (Part B)* 2006; **69**: 1193–1201.

DOI: 10.1002.jlcr