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Human serum transferrin cobalt complex: Stability and cellular uptake of cobalt

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Abstract—Transferrin (Tf) receptor expression is up-regulated on tumour cells. The human serum iron transport protein transferrin (Tf) can bind to many metals including gallium and cobalt. Cobalt has a positron-emitting isotope with a half-life of 18 h and would thus be a useful isotope for imaging purposes. This study has examined the stability of the Co–Tf in the presence of serum and albumin and the uptake of radioactive Co from Co–Tf by tumour cells. Dialysis of ^{57}Co–Tf with serum or with apo-Tf resulted in loss of most ^{57}Co from the complex. The time course of Co uptake from cells incubated with Co–Tf showed an initial rapid association with cells, then a slower rate of accumulation, that is, a similar uptake profile to that of iron. Competition and displacement experiments showed that uptake specifically occurred by interaction with Tf receptors.

1. Introduction

Iron is transported in the blood co-ordinately bound to transferrin (Tf), a 80 kDa protein with a serum concentration of about 35 μM. Transferrin has two iron binding sites, known as the C and the N lobes, each capable of binding to one atom of iron. When the binding sites are empty the protein is described as apo-Tf and when occupied as holo-Tf. Metal binding involves co-ordination with two tyr, an asp, a his and a bidentate carbonate.

Tumour cells have a high demand for iron, so they exhibit enhanced Tf receptor expression.[1] Studies have shown that holo-Tf tagged with radioactive atoms, for example, ^{99m}Tc[2] or with chemotherapy agents,[3] are useful in imaging or tumour treatment, respectively. In addition to iron, Tf can also bind to many other metals[4] and since about 70% of Tf in serum is in the apo form, it has been suggested that Tf may be responsible for the accumulation of metals such as Ga by cancer cells.[5]

Cobalt has a gamma emitting isotope ^{57}Co and a positron-emitting isotope ^{55}Co; the latter has a favourable half-life for imaging ($t_{1/2}$ = 18 h). Co^{2+} can be oxidised by H_2O_2 in the presence of citrate to the 3+ oxidation state. Upon addition of this to a solution of apo-Tf, in the presence of bicarbonate, Co^{3+}–Tf is formed.[6] Limited studies of Co^{3+}–Tf have been carried out[6,7] but many questions remain regarding the properties of this complex. How stable is the complex in serum and does the predominant serum protein, albumin, which has metal chelating properties, remove Co from Co–Tf? Does Co–Tf recognise the Tf receptor? Is Co, when co-ordinately bound to Tf, released by the complex after internalisation as is Fe^{3+},[8] Ga^{3+}[8] and Ti^{4+}[9] but not Zr^{4+}?[10] This paper has attempted to address these questions, which are particularly important when considering Co–Tf as a potential tumour-imaging agent.

2. Materials and methods

2.1. Preparation of Co^{3+}–Tf complex

To 5 mg of $CoCl_2$, dissolved in 30 μl of 1.2 M citrate buffer, pH 5.8, 5 μl of 2 M H_2O_2 was added and left for 45 min during which the solution turned a deep blue colour due to oxidation of Co^{2+} to Co^{3+}. H_2O (170 μl) was then added to the solution. Five milligrams of human serum apo-Tf was dissolved in 1 ml of 10 mM TRIS buffer, pH 9.0, to which 10 μl of 0.5 M $NaHCO_3$ was added. A 100 μl sample was removed and diluted to 1 ml with phosphate buffered saline (PBS) for a blank. Requisite quantities of the Co^{3+} solution were

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added to the remaining apo-Tf solution (containing 50 mM $NaHCO_3$) to attain a 10:1 Co^{3+} to aTf ratio.[6]

The solution was left for 60 min to achieve complexation. The time of complexation was determined by measuring the absorbance of the solution, between 240 and 500 nm compared with the blank containing only the apo-Tf. The solution was then washed to remove non-complexed metal by filtration through a Centricon filter with a 30 kDa cut off. To prepare transferrin labelled with ^{57}Co, 1 MBq of $^{57}CoCl_2$ (Sp Act: 148 MBq/μg Cobalt) was added to 10 μl of 1.2 M citrate buffer, pH 5.8, and 1 μl of 2 M H_2O_2. After addition of 50 μl H_2O the solution was mixed with 1 mg of aTf, incubated at room temperature for 60 min, then washed in a Centricon filter as for the preparation of non-radioactive Co–Tf.

2.2. Stability of Co–Tf and ^{57}Co–Tf

As the molecular weight of Tf is similar to that of albumin they are difficult to separate, so two methods were used to determine the stability of Co–Tf in the presence of albumin and serum. One used spectrophotometry with albumin as a blank, the other dialysis to keep Co–Tf and serum apart.

Co–Tf (0.2 mM) was incubated for 24 h with 1 mM albumin and with 100 mM EDTA. The solutions were then made up to 1 ml and the absorbance measured at 405 nm using water or albumin as a blank.

^{57}Co–Tf (0.2 MBq) was placed in dialysis cartridges (Sigma chemicals, Poole, UK) with a molecular weight cut off of 10 kDa and dialysed in universal tubes containing (a) PBS + 30 mM $NaHCO_3$, (b) serum or (c) 2 mg/ml human apo-Tf in PBS + 30 mM $NaHCO_3$ and incubated at 37 °C for 48 h. Periodically, samples were removed from the incubating medium and counted on a gamma counter.

2.3. Cell studies

MCF7 breast tumour cells were grown in Dulbecco's Modified Eagle's Medium with Glutamax supplemented with 10% foetal bovine serum and 10,000 units/ml of penicillin–streptomycin. For binding and uptake experiments, cells were seeded in 25 cm^2 tissue flasks at 5×10^5 cells per flask and incubated for 3 days at 37 °C. Prior to incubation with ^{57}Co–Tf, the cells were washed 3× with 5 ml of PBS. To determine the time course of incorporation of ^{57}Co into cells, they were then incubated with Medium199 containing 25 mM HEPES buffer (in the absence of serum) and about 0.5 μM Tf labelled with ^{57}Co (20 kBq/ml) for between 2 min and 2 h at 37 °C. To determine the specificity of binding of Co–Tf to Tf receptors, flasks of washed cells were cooled to 4 °C, then incubated for 15 min with medium containing about ^{57}Co–Tf (20 kBq/ml) at 4 °C with or without 100 μM of iron-saturated holo-Tf. Some flasks of cells incubated without iron-saturated Tf were then incubated for 15 min at 37 °C with 100 μM iron-rich Tf to determine if the bound ^{57}Co–Tf was displaced.

3. Results and discussion

3.1. Time of complex formation

Figure 1 shows the change in absorbance between 240 nm and 500 nm of aTf as complexes with Co^{3+} between 1 min and 60 min after addition of Co^{3+}. In agreement with other studies of Co^{3+} and Tf during complex formation,[6,11] absorbance peaks at 247, 295 and 405 nm were observed, which increased with time between 1 and 45 min.

To determine the stability of the complex in the presence of albumin, as this is the predominant protein in serum, the complex was incubated with albumin for 24 h at 37 °C and the absorbance at 405 nm determined using a blank containing the same concentration of albumin. Co–Tf was also incubated with EDTA, a strong chelator of Co^{3+}. Figure 2 shows that incubation with albumin resulted in a decrease in absorbance at 405 nm of about 50%, suggesting that half of the Co^{3+} from Co–Tf had been removed. However, the remaining Tf-bound Co^{3+} was not removed even with a large excess of EDTA. This could be due to differences in the strength of binding of Co^{3+} to the N and C lobes of Tf. Previously,[6] it

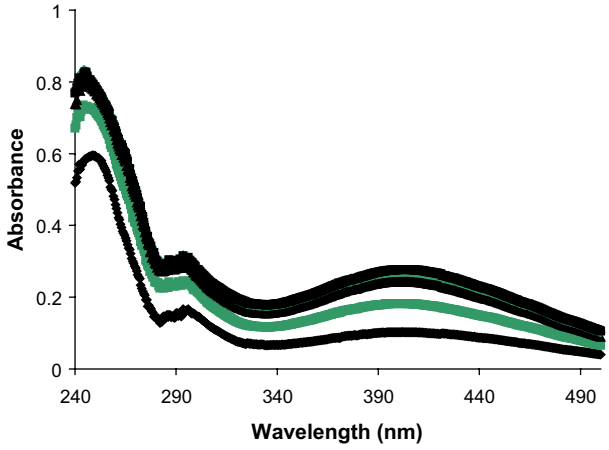

Figure 1. Time of incubation of apo-Tf with a 10-fold molar excess of Co^{3+} (lowest curve 1 min to uppermost 60 min).

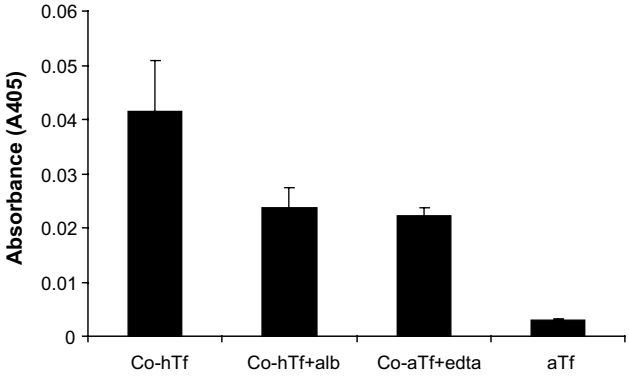

Figure 2. Change in absorbance of 0.2 mM Co–Tf incubated for 24 h with 2 mM albumin or 100 mM EDTA ($n = 4$ measurements for each condition).

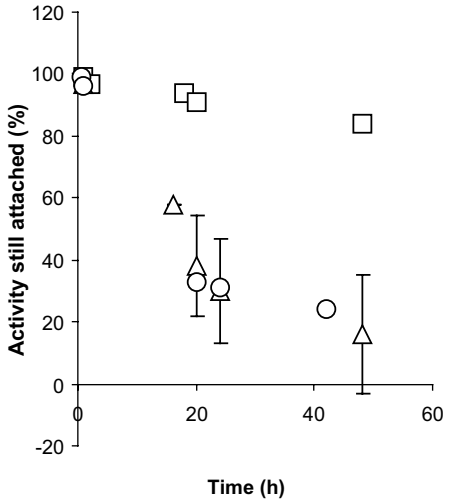

Figure 3. Stability of ^{57}Co–Tf in dialysis cartridges ($n = 3$) incubated in PBS (+30 mM $NaHCO_3$) (squares), serum (triangles) or apo-Tf (circles).

has been shown that Co–Tf when incubated with pyrophosphate, which induces metal release from holo-Tf, does not become fully colourless suggesting only partial loss of Co^{3+}.

Figure 3 shows the loss of activity from dialysis capsules, containing Tf labelled with $^{57}Co^{3+}$, incubated in either PBS, serum or in a solution of apo-Tf over a 48 h period. During the first 24 h of incubation about 70% of activity was lost from the capsules incubated in serum or apo-Tf, while only about 10% of activity was lost from the capsules incubated in PBS.

Incubation of ^{57}Co–Tf with 10 mM EDTA resulted in loss of 81% of the ^{57}Co from the Tf after 24 h at 37 °C. The greater loss of ^{57}Co from ^{57}Co–Tf, than of non-radioactive Co from Co–Tf observed in the spectroscopy experiments may be due to differences in the ratio of metal to aTf used during the labelling procedure. Thus, radio-labelling involved incubation of ^{57}Co with an excess of aTf, whereas Co was in excess when non-radioactive Co–Tf is formed. Due to the trace quantities of radioactive metals used in radio-labelling, the substance to be labelled generally needs to be in excess, so relatively weak binding of radioactive Co with Tf may be inevitable.

Figure 4 shows the uptake of ^{57}Co by tumour cells incubated with medium 199 (with 25 mM HEPES) containing ^{57}Co–Tf (the complex was found to be stable in Medium199 for the duration of the longest incubation period). The time course for the uptake of ^{57}Co by cells incubated with ^{57}Co–Tf shows a similar profile to that of iron[8] with a very rapid association with the cells at early time points, due to receptor binding, then a slower rate of accumulation as the ^{57}Co–Tf is internalised, the ^{57}Co released into the cell, and the receptors recycled allowing other ^{57}Co–Tf molecules to bind. This is in contrast to Tf covalently labelled with ^{99m}Tc where the uptake reaches a plateau when all the receptors are occupied.[2] Not all metals that form complexes with Tf

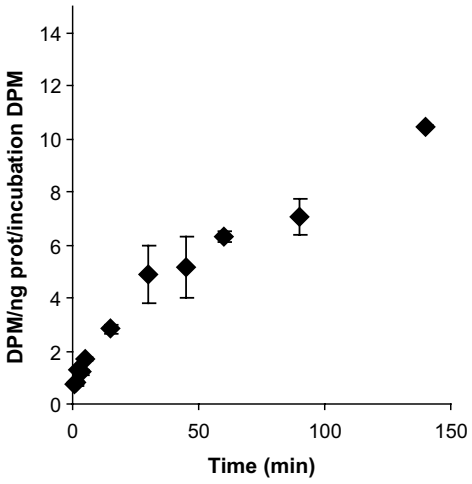

Figure 4. Time course for ^{57}Co incorporation into MCF7 cells incubated with ^{57}Co–Tf.

are released into cells. Thus, complexation of Zr with Tf prevents lobe closure from occurring and this appears to prevent the complex from interacting with the Tf receptor thereby preventing its cellular accumulation.[10]

Incubation of cells for 15 min at 4 °C with ^{57}Co–Tf in the presence of a 200-fold excess of iron-rich Tf reduced the binding of ^{57}Co–Tf by >95%. Figure 5 also shows that incubation of cells with the iron-rich Tf at 37 °C for 15 min after pre-incubating them with the complex for 15 min at 4 °C resulted in the loss of >95% of the bound activity. These results suggest that the complex binds to the Tf receptor.

Iron is also transported into cells by a Tf-independent mechanism.[12] This involves reduction of Fe^{3+} to Fe^{2+} by ferroreductase. The reduced iron is then transported across the membrane. This mechanism also contributes to the cellular accumulation of ^{67}Ga.[13] To examine the relative efficiency of cellular accumulation of Co^{3+} by the Tf transport mechanism with that of a Tf-independent system, cells were incubated with either ^{57}Co–Tf or an equal amount of ^{57}Co as citrate. Figure 6 shows

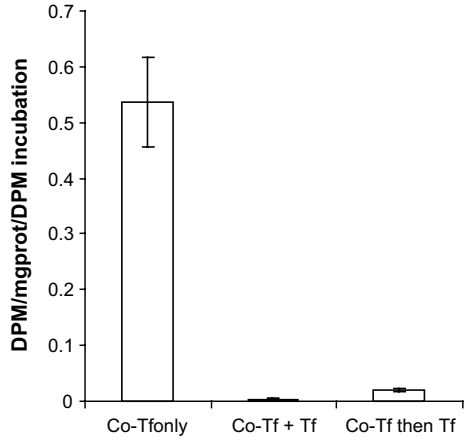

Figure 5. Incubation of ^{57}Co–Tf with MCF7 cells with 200-fold excess of iron-rich Tf or followed by incubation with 200-fold excess of iron-rich Tf ($n = 3$).

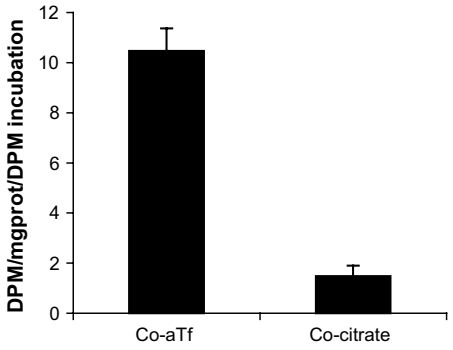

Figure 6. Uptake of ^{57}Co by cells incorporated with ^{57}Co–Tf or with ^{57}Co citrate ($n = 4$).

that there is clearly a much greater rate of uptake of radioactivity by cells incubated with ^{57}Co–Tf than with ^{57}Co-citrate.

The binding constant for Co^{3+} to Tf is unknown probably due to the scarcity of known binding constants of Co^{3+} with other chelating agents from which that of Co^{3+}:Tf could be derived by titration. However, it has been predicted to be similar[4] to that of iron, that is, about log 22. The complex is stable in PBS but when dialysed using serum or apo-Tf most of the $^{57}Co^{3+}$ is lost from the original complex within 24 h. Despite the high binding constant for Ga^{3+} with Tf of about log 20,[14] Ga readily dissociates from Ga–Tf even in saline solution.[14,15] However, Ga has been used for many years as a tumour-imaging agent and Co^{3+} when complexed with Tf may efficiently accumulate in tumour tissue even though it is likely to exchange with other Tf molecules in serum and some possibly with albumin. Given the efficient accumulation of cobalt by tumour cells incubated with ^{57}Co–Tf and the existence of ^{55}Co, a positron-emitting metal with a favourable half-life for imaging purposes, its imaging potential should be examined further.

Acknowledgments

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