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COUPLING OF ^{125}I-EGF TO SERUM ALBUMIN FOR THE STABILIZATION OF RADIOIODINE BOND*

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Summary: Radioiodinated epidermal growth factor (^{125}I-EGF) used for the detection of EGF receptors in implanted tumors in mice showed rapid in vivo de-iodination. To increase its stability it was coupled to mouse serum albumin by cyanuric chloride method with > 60 % efficiency. ^{125}I-EGF and albumin coupled EGF were purified by HPLC. ITLC analysis with saline as a solvent indicated the in vitro stability of both of the radioiodinated products up to 8 days of testing. Biodistribution of ^{125}I-EGF in normal mice showed rapid de-iodination, fast clearance from the blood by urinary excretion and high thyrodial uptake. High levels of free ^{125}I$^-$ were obtained when plasma and urine samples were analyzed by ITLC. The biological effect of albumin coupling of EGF was demonstrated as increased whole-body retention, prolongation of blood clearance and as lower levels of thyroidal radioactivity.

Radyoiyot Bağını Stabilize Etmek İçin ^{125}I-EGF'in Serum Albuminine Bağlanması

Özet: İmplante tümörlü farelerde reseptör lokalizasyon ve tanımlanmasında kullanılan radyoiyot ile işaretli "epidermal growth factor"ün (^{125}I-EGF) in vivo kararsız olduğu gözlendi. İyot-EGF bağını kararlı hale getirmek için EGF molekülü fare serum albüminine siyanürik klorür metoduyla >% 60 nispetinde bağlandı. ^{125}I-EGF ve albümine bağlı ^{125}I-EGF HPLC ile saflaştırıldı. ITLC analizi her iki maddenin de test süresi olan 8 gün kararlı olduğunu gösterdi. ^{125}I-EGF ile farede yapılan biyodağılım çalışmaları çok hızlı bir şekilde ^{125}I'in açığa çıktığını gösterdi. Kan klirensi ve idrar atılımı hızlı, tiroit tutulumu yüksekti. Plazma ve idrar nümuneleri ITLC ile analiz yapıldığında yüksek düzeyde ^{125}I$^-$ aktivitesi gösterdi. EGF'ün albümine bağlanmasıyla elde edilen biyolojik etki total vücut retansiyonunda artma, kan klirensinde yavaşlama ve tiroit tutulumunda azalma idi.

Key words : *^{125}I-EGF, ^{125}I-EGF-Albumin, EGF receptors, HPLC, ITLC*
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INTRODUCTION

Although ^{131}I does not have the necessary ideal imaging characteristics it is the most often used radioisotope compared to ^{123}I, ^{99m}Tc and ^{111}In in labelling monoclonal antibodies for in vivo studies (1). Its long half-life of 8 days, though detrimental from the standpoint of radiation dosimetry is advantageous when the tumoral uptake is prolonged and a follow-up of one week is necessary (1,2). The in vivo stability of the label may present a serious problem. For metal labels, i.e. ^{111}In, conjugation of a bifunctional chelator such as cDTPA containing an active functional group that can be covalently bound to a biological molecule at one end and a metal binding group at the other end was proposed (3) and found wide application (4,5). For iodine labels, most recently Sinn et al. (6) proposed cyanuric chloride coupling of small radioiodinated compounds to serum albumin used as a carrier molecule in order to enhance the tumor uptake. They observed after coupling prolonged biological half-life, increased accumulation in neoplastic tissues and no significant radioiodine uptake of the thyroid gland giving evidence against in vivo deiodination. They concluded that after cyanuric chloride coupling the hydroxyl group of the phenyl ring was transformed into an ether bridge and this stabilized the neighbouring carbon-iodine bond against cleavage by enzymes.

Epidermal growth factor (EGF) receptors have been demonstrated on cultured breast carcinoma cell lines (7) and biopsy samples from human breast cancers (8,9). We have been using ^{131}I labelled human EGF for the localization of EGF receptors in nude mice implanted with human breast carcinoma xenografts (10). In recent experiments we have observed rapid in vivo deiodination resulting in high blood background, high thyroid uptake and increased urinary excretion with no significant accumulation in tumoral tissues. We thought that coupling EGF to mouse albumin by using the cyanuric chloride method of Sinn et al. (6) might stabilize it against in vivo de-iodination. The present investigation was undertaken to realize the coplung of ^{125}I-EGF to albumin, to find the in vitro and in vivo stability and biodistribution of ^{125}I-EGF-Alb in normal mice compared to ^{125}I-EGF.

MATERIALS AND METHODS

Human Epidermal Growth Factor (EGF) (M.W = 6348.2) was bought from Bissendorf Biochemical gmbh, Hannover. Cyanuric chloride and mouse albumin were bought from Sigma Chem. Co. (U.S.A.), Iodogen was purchased from Pierce Chem. Co., U.S.A. ^{125}I for protein iodination and ^{125}I-EGF were purchased from Amersham, England.

Labelling EGF with ^{125}I and Coupling to Albumin: The labelling was carried in Eppendorf cups containing iodogen. Iodogen was dissolved in chloroform and 0.2 ml containing 20 µg iodogen was placed in each cup. The cups were dried under a stream of nitrogen and stored at -20°C until use. EGF, cyanuric chloride and albumin solutions were prepared fresh just before reaction in the following way: 130 µg EGF which is 77.1 % EGF was dissolved in 50 µl distilled water to give 2 µg/µl solution. 10 mg cyanuric chloride was dissolved in 1,4-dioxane to give 1

mg/ml solution. 2.5 mg mouse albumin was dissolved in 37.5 µl phospate buffer (0.5 M, pH=9).

The reaction was carried out at room temperature in the following sequence:

17 µl EGF solution (33 µg EGF= 5.17 nmol) was placed in an iodogen cup. 50 µl phospate buffer (0.5 M, pH= 7.5) and 4 µl ^{125}I containing 74 MBq radioactivity were added.

The cup was vortexed for a few sec and left to react for 2 min.

The reaction mixture was transferred to another Eppendorf cup which did not contain iodogen in order to stop the reaction.

At this point ^{125}I labelled EGF was obtained at an efficiency of ~90 %. EGF was coupled to albumin in the following steps:

2 µl cyanuric chloride solution (2 µg=10.8 nmol) was added.

The cup was vortexed for a few sec and left to react for 2 min.

10 µl albumin solution (660 µg=10.2 nmol) was added immediately.

The cup was vortexed for a few sec and left to react for 30 min.

The whole amount (83 µl) of the reaction mixture was injected to the HPLC column described below for the analysis and purifaction of ^{125}I-EGF-Alb. ^{131}I-EGF was also purified this way.

HPLC Purification of ^{125}I-EGF and ^{125}I-EGF-Alb: The HPLC apparatus (LKB) used had the following characteristics:

High pressure pump: Pharmacia-LKB, Model: 2150

Rheodyne injection valve

Variable U.V. detector: Pharmacia-LKB, Model: 2151

Recorder: Pharmacia-LKB, Model: 2210

Fraction collector: Pharmacia-LKB, Model: 2211 Superrac

Column: Pharmacia-LKB, Superose 12, 300x10 mm

Eluent: Phopspate buffer (0.05 M, pH= 7.0)

Flow: 0.5 ml/min

U.V.: 254 nm

Fractions: 0.5 ml.

Mouse albumin (66.7 µg/µl phosphate buffer), unlabelled EGF (2 µg/µl), ^{125}I-EGF (33 µg in 71 µl) and ^{125}I-EGF-Alb (83 µl), and free $^{125}I^-$ were all analyzed by HPLC separately. The radioactivities of the fractions collected were determined in a dose calibrator (Isotope Calibrator II, Berthold) and plotted on the corresponding curves obtained by the UV detector. The fractions corresponding to ^{125}I-EGF were combined and diluted with saline to give 740 kBq/0.2 ml for animal experiments. To stabilize ^{125}I label mouse albumin was dissolved in this solution to a concentration of 2 %. ^{125}I-EGF-Alb. fractions were also combined and diluted to the same concentration without further addition of albumin.

Chromatographic Quality Control: Impregnated-Thin-Layer-Chromatography (ITLC) was utilized with ITLC-SG mini-strips (Gelman Instruments, U.S.A.) and saline as a solvent

to determine the amount of free $^{125}I^-$ in the labelled products (11). Free iodide migrated with the solvent front (Rf= 1.0) while radioiodinated compounds remained at the origin (Rf= 0.0). ^{125}I-EGF before and after passage through HPLC column, ^{125}I-EGF-Alb. after column, serum and urine samples of mice injected with either of the labelled substances, obtained at different intervals were all analyzed as to the amount of free $^{125}I^-$. The stabilities of Amersham's and in-house labelled ^{125}I-EGF and ^{125}I-EGF-Alb were checked at certain intervals up to 8 days by the same chromatographic method.

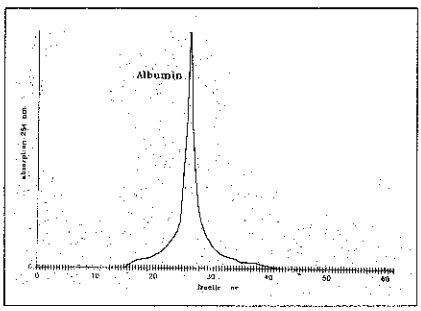
Animal Studies: 25 NMRI mice were each I.V. injected with 0.2 ml (740 kBq) ^{125}I-EGF obtained from Amersham through the tail vein. They were sacrificed in groups of 5 at 1, 3, 6, 12 and 24 h post-injection. Some blood, thyroid, lungs, liver, kidneys, spleen femur, muscle and stomach were removed. Some of the blood was centrifuged to separate the serum for ITLC analysis. They were all weighed and counted against a standard prepared from 1/100 dilution of the injected solution in a gamma counter (Model: BF 5300, Berthold, Germany). Percent uptake by each organ and per gram tissue and the means with standard deviations of 5 animals were all calculated. The same procedure was followed with in-house prepared ^{125}I-EGF. With ^{125}I-EGF-Alb the only difference in the procedure was in the time of sacrifice of the animals which were 1, 3, 6, 24 and 48 h post-injection.

The radioactivities of the whole animals were determined at the same intervals up to the time of sacrifice by the use of a whole-body counter against a standart having 100 % of the injected solution. % retention of radioactivity was calculated for each animal and the mean values were plotted as a function of time.

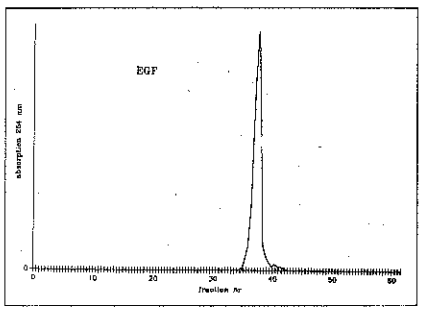
RESULTS

EGF was labelled with ^{125}I with high efficiency by the iodogen method. The amount of free $^{125}I^-$ was ~ 10 % before and 2.3±1.2 % after passage through HPLC column. The in vitro stability was comparable to Amersham's product up to 8 days of testing. There was 4.1±2.4 % and 2.9±1.1 % free ^{125}I in Amersham's and in-house prepared ^{125}I-EGF, respectively. ^{125}I-EGF-Alb was also stable in vitro in the interval of 8 days (2.5±1.2 % free $^{125}I^-$). Figure 1 shows the HPLC chromatograms for the cold and radioiodinated producuts. The maximum of the mouse albumin peak appeared at fraction 26 and that of EGF at fractions 37-38 (Fig. 1 a and b). EGF gave a sharper peak than albumin, indicating its high level of purity. ^{125}I-EGF had the same maximum as the cold EGF (Fig. 1 c). After coupling reaction, in addition to albumin and EGF peaks a sharp peak was obtained at fraction 24 by UV absorption that did not contain radioctivity. It might be albumin dimer occuring as a result of cynanuric chloride coupling. Two main radiocativity peaks were obtained corresponding to Alb and EGF fractions. Albumin peak was broader compared to EGF. This peak indicated that coupling was realized (Fig. 1 d). 60-65 % of ^{125}I-EGF was used up in the coupling reaction. In order to obtain a highly pure sample for the animal studies we took only the fractions 26-28 and combined them to have a sample containing 30-35 % of the total radioactivitiy. Free ^{125}I appeared at fractions 40-42.

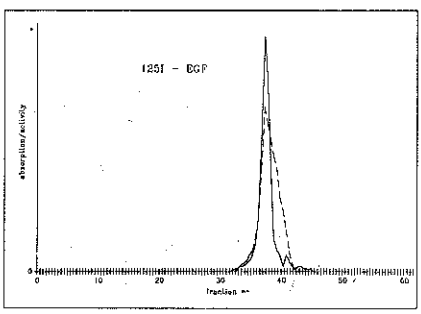
Figure 1. Typical chromatograms obtained with HPLC by UV detection at 254 nm (solid line) and radioactivity measurements (broken line):



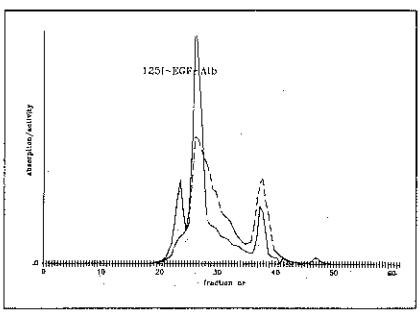
(a) mouse albumin



(b) cold EGF



(c) ^{125}I-EGF



(d) ^{125}I-EGF-Alb

Results of biodistribution studies in mice demonstrated no significant difference between Amersham's and in-house labelled ^{125}I-EGF. Table 1 summarizes the results obtained with our ^{125}I-EGF. Radioactivity levels in all the organs decreased in parallel to the blood clearance except for thyroid activity which reached a maximum at 6 h, indicating the presence of free $^{125}I^-$ in vivo. Biodistribution of ^{125}I-EGF-alb is given in Table 2. The results obtained with coupled ^{125}I-EGF are different from the uncoupled product. The blood clearance as well as the disappearance of radioactivity from all the organs were prolonged. The thyroidal radioactivity

Table 1. Biodistribution of ^{125}I-EGF in normal mice (%/g) tissue).

	Time (h)				
Organ	1	3	6	12	24
Blood	5.53±1.63	2.87±1.42	0.984±0.411	0.0811±0.0123	0.0614±0.0099
Serum	6.16±1.54	3.11±1.46	1.09±0.50	0.0650±0.0176	0.0565±0.0176
Thyroid	140.8±59.0	206.7±19.2	1045.2±376.4	320.9±276.1	125.9±93.1
Lungs	3.41±1.01	1.80±0.7	0.666±0.291	0.0799±0.0637	0.0439±0.0094
Liver	4.46±2.13	1.02±0.45	0.396±0.119	0.0558±0.0171	0.0327±0.0086
Kidneys	7.05±1.56	2.49±0.90	0.983±0.235	0.185±0.031	0.0855±0.0500
Spleen	2.29±0.65	0.867±0.740	0.394±0.115	0.0578±0.0152	0.0488±0.0050
Femur	1.76±0.56	0.943±0.425	0.364±0.099	0.0566±0.0032	0.0499±0.0155
Muscle	0.86±0.31	0.586±0.291	0.196±0.034	0.0282±0.0082	0.0359±0.0068
Stomach	3.28±0.91	2.13±1.11	0.718±0.227	0.0716±0.0406	0.0460±0.0048

Table 2. Biodistribution of ^{125}I-EGF-Albumin in normal mice (%/g tissue)

	Time (h)				
Organ	1	3	6	24	48
Blood	14.17±0.57	9.29±1.15	6.97±0.32	3.58±0.15	2.22±0.40
Serum	27.75±0.88	18.49±1.69	14.28±0.91	7.07±0.18	3.84±0.53
Thyroid	7.11±0.93	17.69±5.39	24.72±2.11	47.65±39.08	23.47±22.30
Lungs	5.23±0.19	3.63±0.14	3.03±0.31	1.35±0.16	0.949±0.148
Liver	18.14±0.83	14.35±1.5	10.82±0.89	6.16±0.24	2.36±0.27
Kidneys	12.19±0.29	8.92±0.32	6.45±0.49	2.83±0.086	1.62±0.53
Spleen	8.99±1.54	4.56±0.60	2.67±0.27	1.33±0.085	0.774±0.152
Femur	2.50±0.12	1.85±0.16	1.34±0.16	0.726±0.0138	0.241±0.0672
Muscle	0.793±0.153	0.684±0.120	0.608±0.048	0.359±0.0477	0.525±0.208
Stomach	1.53±0.11	1.51±0.30	1.15±0.037	0.717±0.148	0.313±0.052

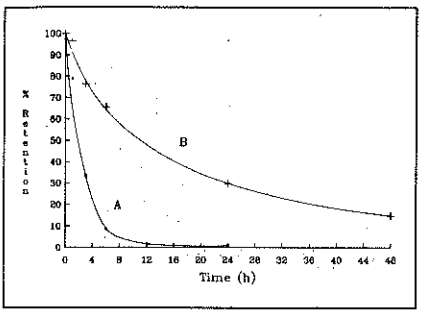


Figure 2. Whole-body retention of ^{125}I-EGF (A) and ^{125}I-EGF-Alb (B) in normal mice.

was much smaller in comparison to ^{125}I-EGF. The amount of free $^{125}I^-$ in serum samples were higher with ^{125}I-EGF compared to ^{125}I-EGF-Alb (Table 3). In urine samples 59.6 % and 56.5 % free $^{125}I^-$ were obtained with Amersham's and our ^{125}I-EGF, respectively, within 3 h post-injection. The radioactivity in urine samples of ^{125}I-EGF-Alb injected mice was not sufficient for chromatographic analysis. The whole-body retention of ^{125}I-EGF-Alb was higher in contrast to ^{125}I-EGF (Fig 2).

DISCUSSION

In the present investigation ^{125}I-EGF was coupled to serum albumin with a high total efficiency of > 60 % in a simple and rapid procedure. This is the first time a large molecule, a peptide such as EGF, is coupled to a large protein molecule such as albumin (Alb/EGF M.W. ratio is ~ 10/1). The radioiodinated compounds used by Sinn et al. (6) were of smaller molecular weight. During coupling reaction a variety of other prodcuts are also expected, such as EGF-EGF, $(EGF)_2$-Alb, EGF-$(Alb)_2$ and some separate and mixed polymers of both with cold and radioactive EGF increasing the number of alternatives. Degradation of either EGF and albumin might also occur and complicate the results further. However, under the conditions presented in this paper, EGF-Alb is favored to others. We used twice the molar amount of albumin and cyanuric chloride compared to EGF. We observed hydrolysis of cyanuric chloride after addition to the aqueous iodination mixture. Consequently 1:1 molar ratio was not sufficient for the coupling reaction. In amounts greater than 2:1 larger molecular weight

Table 3. Amount of free $^{125}I^-$ iodide in serum samples of mice injected with ^{125}I-EGF, determined by ITLC-SG strips and saline as a solvent expressed as % present in serum samples.

	% $^{125}I^-$ (*mean±S.D.)				
	1h	3h	6h	12h	24h
1. ^{125}I-EGF from Amersham	20.2±6.5	9.0±2.0	4.5±4.6	8.4±4.7	—
2. ^{125}I-EGF, in-house	44.4±24.6	15.2±7.0	6.3±4.0	10.8±5.3	10.7±10.5
3. ^{125}I-EGF albumin	13.3±7.7	16.4±4.1	9.2±3.3	6.9±3.6	7.8±3.5

* Each point is a mean of 5 determinations

products were obtained on HPLC analysis. Addition of albumin in twice the amount of EGF was necessary to prevent the formation of other products such as EGF-EGF and polymers. Reaction time of EGF with cyanuric chloride was limited to 2 min, because with longer reaction times a mixture of unidentifiable products was obtained. 30 min of reaction time was necessary to couple the ^{125}I-EGF-cyanuric chloride complex to albumin in order to obtain high coupling efficiency.

The rapid clearance of radioactivity and high thyrodial uptake that we observed with ^{125}I-EGF was also reported for radioiodinated somatostatin analogue used in the detection of somatostatin receptors in tumor bearing rats (12). The biological effect of albumin coupled EGF was demonstrated in normal mice as increased whole-body retention, prolongation of blood clearance and decreased thyroidal uptake. The effect on tumoral uptake will be investigated in nude mice in a further study. Cyanuric chloride coupling reaction can be used for other radioiodinated compounds, peptides, of biological significance as a practical solution againt in vivo cleavage of iodine-carbon bond by enzymes, thus increasing the in vivo stability of the molecule. The longer half-time of Alb coupled EGF in blood might increase its tumoral uptake if it has slow kinetics. On the other hand, it is also probable that the receptor specific sites on EGF molecule might be rendered inaccessible as a result of coupling with a very large molecule. Studies with nude mice with implanted tumors will give evidence as to the biological efficacy of the present method.

In conclusion, our results indicate that it is possible to couple a large peptide to a stable protein molecule to protect the radioiodine bond against in vivo enzymatic cleavage, thus prolonging its biological half-time. The method can be applied to other unstable peptides.

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İnsanların duyguları, bilgileriyle ters orantılıdır. Ne kadar az bilirseniz, onu o kadar şiddetle savunursunuz.

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