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Preparation and Purification of an End to End Coupled mEGF−Dextran Conjugate

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The amino terminus of mouse epidermal growth factor (mEGF) was coupled directly to the aldehyde end of dextran through a reductive amination procedure. The highest coupling efficiency was ∼80% and could be reached after ∼24 h of reaction time at pH 8. Gel filtration on Sephadex G-50 Fine removed free mEGF from the conjugate. Preparative polyacrylamide gel electrophoresis was used to separate the conjugate from excess noncharged dextran. The conjugate bound specifically to the EGF receptor on cultured glioma cells as shown in displacement tests with free mEGF. The conjugate was stable in the pH interval 4−9, in 2 M sodium chloride, in 7 M urea, and in human serum and could still bind to the EGF receptor after such treatments. The conjugates are candidates for targeted nuclide therapy.

INTRODUCTION

Dextran has for a long time possessed biomedical interest, primarily as a blood plasma volume expander (DeBelder, 1990; Fassio et al., 1992; Klotz and Kroemer 1987; Moon et al., 1991) but also as a carrier for drug and radionuclide (Andersson et al., 1991; Carlsson et al., 1994; Olsson et al., 1994; Sjöström et al., 1997) delivery. An attractive approach is to couple dextran to a ligand that recognizes a specific receptor on cancer cell membranes. The dextran part is then the potential carrier of the toxic factors such as radionuclides. Targeted therapy with radionuclides for cancer treatment is one main research topic in our laboratory and is therefore discussed in more detail.

Dextran connected to a tumor seeking ligand has potential advantages when used as a radionuclide carrying conjugate for the following reasons:

- There is a reduced risk for biodegradation since dextran has been shown to protect against protein degradation (Andersson et al., 1991; Kato et al., 1990; Marshall, 1978; Srivastava, 1991). The dextran moiety could possibly protect the conjugate from intracellular degradation (Andersson et al., 1991; Blomhoff et al., 1983; Olsson et al., 1994; Sjöström et al., 1997) and also from degradation in the blood circulation (Melton et al., 1987; Yasuda et al., 1990).
- Dextran has potentially high toxicity when loaded with toxic agents but low toxicity due to the EGF and dextran structures since neither dextran (DeBelder, 1990) nor the natural ligand EGF is expected to be toxic. The normal ligand epidermal growth factor (EGF)[1] is known to react only with the EGF receptor and have no known cross-reactivity with other structures (Carpenter, 1987). Thus, EGF is not expected to exert any unwanted blocking of enzymatic reactions or to disturb other cellular processes.
- Control of serum half-life of the conjugate is possible by varying the molecular weight of the dextran. The length of the dextran chains will probably, to a large extent, determine the serum half-life (Emmrich et al., 1977; Klotz and Kroemer, 1987; Lindström et al., 1993).

Furthermore, it is possible that there is a decreased risk for immunogenic reactions against the conjugate in relation to the protein itself. Theoretically, it is possible that the antigenicity could be reduced since the carbohydrate part might "hide" immunogenic sites on the ligand. However, mEGF is a relatively small protein (6 kDa) and such molecules are normally not strongly immunogenic, so this might not be an important factor when EGF−dextran is considered. Dextran is not known to be immunogenic except in those few cases when persons have an immunology-based hypersensitivity to dextran (Klotz and Kroemer, 1987). However, the increased molecular weight of the conjugate in relation to the individual "building blocks" might increase the antigenicity. It is also a risk that new structures appearing in the conjugation point between EGF and dextran might be immunogenic, and this has to be tested in future animal experiments and clinical tests. The situation is complex as shown in other similar cases (Seppala and Makela, 1989), and the question about immunogenic response is important for future therapeutic applications since it might be necessary to give repeated injections of the targeting agent.

These arguments support the ambition to further analyze the basic aspects of dextran−ligand conjugates for targeted radiotherapy. However, each aspect has to be considered with great care since the conjugates will be hybrid molecules consisting of a carbohydrate part, dextran, and a protein, EGF, and such hybrid molecules might attain new unpredicted biological properties.

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[1] Abbreviations: CDAP, 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate; PB, phosphate buffer; PBS, phosphate-buffered saline (at pH 7.2 and 0.15 M PBS); EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; mEGF, mouse epidermal growth factor; Dex, dextran D14.

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The receptor for EGF is overexpressed in some malignancies such as gliomas, bladder cancers, squamous epithelial carcinomas, and some forms of adenocarcinomas (Bigner et al., 1988; Carpenter, 1987; Filmus et al., 1985; Libermann et al., 1984, 1985; Mendelsohn, 1988; Messing et al., 1992; Ozawa et al., 1989; Sauter et al., 1994; Todderud and Carpenter, 1989) and is a potential target for therapy in those malignancies. The goal with the present study was to design a reasonably well-defined EGF receptor targeting agent, based on the natural ligand EGF conjugated to dextran, for the future delivery of radioactive nuclides of therapeutical interest.

EGF is a 53 amino acid protein with compact structure that binds specifically to the EGF receptor. Human and mouse EGF have 37 of 53 amino acids in common, and the three disulfide bonds are formed in the same relative positions. They have also approximately the same binding efficiency to the human EGF receptor (Carpenter and Cohen, 1976, 1979). We decided to use mouse EGF in the present study since it only has one free amino group, namely the amino terminus (Carpenter and Cohen, 1979). Human EGF has, in addition to the amino terminus, also two lysine residues. Thus, compared to human EGF, mouse EGF might be more suitable for specific amino group coupling to dextran.

Many different protein−dextran conjugates have been developed with various methods. The coupling methods often employed are periodate oxidation of dextran and CNBr or CDAP carbohydrate cyanylating procedures (Andersson et al., 1991, 1992; Holmberg et al., 1993, 1994; Lövqvist et al., 1993; Olsson et al., 1994; Sjöström et al., 1997). These methods can effectively introduce free amino group containing substances at random positions in the dextran chain. The main disadvantage seems actually to be that the positions on the dextran for linking of the protein are randomly distributed, which gives a heterogeneous mixture of conjugates.

From the view of drug design it would be interesting if the coupling between the tumor seeking ligand and dextran could be limited to one "specific position" in the dextran chain. This could give a more homogeneous conjugate preparation. There seems to be only one such specific position in dextran, and that is the aldehyde group at the end of the dextran chain. Only one such aldehyde group exists per dextran molecule even if some branching exists (Fudui et al., 1982).

The principle mechanisms for selective reductive amination of aldehyde groups have previously been described (Borch et al., 1971; Lane, 1975; Lundblad and Noages, 1984), and some results for coupling of small amino containing molecules to the end aldehyde group have also been described earlier (Yalpani and Brooks, 1985). In the present study we have investigated the possibility of using selective reductive amination of the single aldehyde group of dextran to directly couple to the amino terminus of mEGF.

EXPERIMENTAL PROCEDURES

Chemicals. Freeze-dried dextran with a molecular range 14 000 ± 500 was obtained by fractionation of Dextran T10 (Pharmacia Biotech AB, Uppsala, Sweden) by gel filtration on Sephacryl 100 HR (Pharmacia Biotech). This dextran fraction will in the following be called D14. Sodium cyanoborohydride ($NaCNBH_3$) (article no. 818053) was the product of Merck-Schuchardt, Darmstadt, Germany. Mouse EGF (batch 57.067), tissue culture grade, was purchased from Janssen Biochimica, Belgium. ^{125}I (article no. IMS 30) was from Amersham Laboratories, England.

Equipment. Sephadex G-50 Fine, FPLC System, and PhastSystem are products of Pharmacia Biotech AB. The instrument for preparative eletrophoresis, a Mini Prep Cell, was from Bio-Rad Inc., Richmond, CA. A Beckman refractomonitor was used for continuous registration of the refractiive index during chromatography. A Wallac 1480 Wizard gamma counter (Wallac, Finland) was used to count ^{125}I radioactivity. The electronic cell counter was from Coulter Electronics LTD, England.

Radiolabeling. EGF was labeled with ^{125}I according to the chloramine-T method as previously described (Andersson et al., 1991). mEGF (2.5−5 μg) was mixed with 20 μL of pH 7.5, 0.5 M, PB, 10 μL of 0.2% (w/v) chloramine-T, and 10−20 μL of ^{125}I for 1 min. Twenty-five microliters of 0.2% (w/v) sodium bisulfite solution was used to stop the reaction. Excess reagents and free ^{125}I were separated, directly after labeling, from the macromolecules by NAP-5 desalting columns (Pharmacia Biotech AB). Buffer with pH 8.0, 0.05 M, PB was used as washing solution.

Reductive Amination. Dextran D14 (20 mg, 1.43 mmol) was dissolved in 100 μL of iodinated mEGF (1−5 μg, 0.17−0.83 nmol) solution (pH 8.0, 0.05 M PB). $NaCNBH_3$ (8 mg, 127 mmol) was then added. The reaction was kept going for 24 h (in the range of 0−145 h in the initial tests) at room temperature with continuous stirring. The reductive amination reaction scheme can be written (Dex = dextran D14)

$$\text{Dex-CHO} + \text{mEGF}-\text{NH}_2 \rightarrow \text{Dex-HC}{=}\text{N-mEGF}$$

$$\text{Dex-HC}{=}\text{N-mEGF} \xrightarrow{\text{NaCNBH}_3} \text{Dex-CH}_2-\text{NH-mEGF}$$

The yield of conjugate production was optimized as a function of the pH of the reaction buffer solution. The reaction conditions were the same as above only that pH values in the range 6.5−8.5 in 0.05 M PB solutions were applied. The uncertainty in each calculated point was estimated from repeated pipettings, variations in the chromatography steps, and Poisson statistics for radioactivity counting. It was then estimated that the error in each measurement was <5%.

Gel Filtration. The reductive amination reaction mixture was applied to a Sephadex G-50 Fine (1 × 40 cm) column. A Pharmacia HR 10/30 column was used in some cases. The flow rate was 0.2 mL/min, and the fraction volumes were 0.5 mL. Eluents were either 0.09% (w/v) sodium chloride, PBS, or distilled water depending on the application. A refractomonitor continuously registered the result of the chromatography, and the fractions were then analyzed with the gamma counter.

Analytical Electrophoresis. A PhastSystem for polyacrylamide gel electrophoresis was used to analyze the obtained conjugate solutions. Native 8−15% gradient gels were loaded with 4 μL samples and the samples run at 400 V for half an hour (268 Vh). The gel film was then cut into 30 equally sized pieces for every sample lane. Each piece was transferred to 0.5 mL of PBS and analyzed with the gamma counter.

Preparative Electrophoresis. A discontinuous native-PAGE system for preparative electrophoresis was used to isolate the conjugate from excess amounts of free dextran (dextran without EGF). The system was as follows: 4% stacking gel (7 × 15 mm) with pH 6.8, 0.125 M, Tris-HCl and 7% resolving gel (7 × 40 mm) with pH 8.8, 0.375 M, Tris-HCl. Tris-glycine buffer (25 mM Tris, 200 mM glycine) was used as the running and elution buffer. Usually 150−250 μL of sample was mixed with the same volume of sample buffer [pH 6.0, 62.5 mM, Tris-

HCl, 25% (v/v) glycerol, 0.012% (w/v) bromophenol blue]. The electrophoresis was then run at 300 V with constant power and current limits at 1 W and 3 mA for 20 h. The elution buffer was pumped at a rate of 100 μL/min. The obtained fractions were finally analyzed with the gamma counter.

Dextran Analysis. The dextran concentration in the collected fractions was determined using the phenol method according to Dubois et al. (1956).

Cell Culture. The subclone of the glioma cell line U-343-MG used in this study was originally designated U-343MGaC12:6 and has been characterized in some detail by Westermark et al. (1982), Nistér et al., (1987), and Werner et al. (1988). Ham's F-10 medium (Kebo, Stockholm, Sweden) supplemented with 10% FCS (Flow, Stockholm, Sweden), L-glutamine (2 mM), and PEST (penicillin 20 IU/mL and streptomycin 20 μg/mL) (all from Kebo) were used. The culture medium was routinely changed three times per week. Subculture with trypsin was applied normally once a week. The cells in the EGF-binding tests were in all cases not trypsinized for <2 days before the tests since trypsinization might damage the EGF receptors. The number of cells in each of the culture dishes (plastic culture dishes with a diameter of 35 mm, Corning Glass, Corning, NY) used for binding experiments was 5×10^5.

Displacement Tests. The capacity of normal nonradioactive mEGF to displace the binding of [^{125}I]mEGF–Dex conjugate was analyzed. Conjugate samples of ~5 μg in 11 mL of F10 medium (complemented with PEST) were prepared. Various concentrations of nonradioactive mEGF in culture medium were used to displace the conjugate binding. The nonradioactive mEGF was given to the cells directly before a constant concentration of radioactive conjugate was added to the culture dishes. In practice, the nonradioactive and radioactive components were give at the same time because the time interval between was only a few seconds. The total medium volume was 2 mL in each dish. The incubations were in all experiments carried out as triplicates. The incubation times were 90 min at 37 °C, which had been found to be suitable for similar types of conjugates in previous studies from our laboratory (Andersson et al., 1991; Olsson et al., 1994; Sjöström et al., 1997).

After incubation, the cultures were washed, 6×1 min, in normal nonradioactive culture medium. The cells were then trypsinized with 500 μL of trypsin EDTA solution [0.05% trypsin (w/v), 0.02% EDTA (w/v) in buffer] for 15 min at 37 °C. One milliliter of normal culture medium was then added to each dish to obtain a homogeneous suspension of cells. A 0.5 mL fraction of the cell suspension was taken for electronic cell counting. The remaining 1 mL volume was analyzed in the gamma counter using the standard ^{125}I channel to measure the cell-associated radioactivity.

Stability Tests. The conjugates were exposed to different conditions to test their stabilities. The universal buffers with different pH values and containing different combinations of NaOH, potassium dihydrogen phosphate, boric acid, and diethylbarbituric acid were applied. The choice of these buffers was made since they are often used in biochemical and biological work. NH_4SCN (3 M), urea (7 M), NaCl (1 M) (all three giving high ion strength), or 30% ethylene glycol (hydrophobic) (w/v) was also applied since these are often used for elution in affinity chromatography. We did not apply affinity chromatography in this study, but that might be the case if conjugate specific antibodies are raised in the future. Human serum was also used in the stability tests. The conjugate preparations were collected from gel filtration on the Sephadex G-50F (1×40 cm) columns, eluted and washed with 0.09% NaCl (w/v). The conjugate fractions were pooled, divided in equally sized fractions, and kept frozen before the tests.

During the stability tests, the [^{125}I]mEGF–dextran conjugate solutions (in 0.09% NaCl) were mixed with the same volume of universal buffer of different pH or with 6 M NH_4SCN, 14 M urea, 60% ethylene glycol (w/v), or human serum, respectively. The mixtures were incubated at 37 °C for 1 h and then immediately applied to Sephadex G-50 F (1×40 cm) gel filtration. A buffer with pH 7.0 (0.05 M PB) was used to elute and wash the column. The ^{125}I radioactivity in each fraction was measured with the gamma counter.

Binding to cultured cells was finally analyzed as described above (under Displacement Tests), and 0.5 mg/mL nonradioactive mEGF was in this case added in the culture medium to displace the binding.

RESULTS

After reductive amination, the conjugates were freed from nonreacted EGF by gel filtration on Sephadex G-50 Fine. When a mixture of mEGF–dextran conjugate, mEGF, and dextran D14 was applied to the column, three peaks were obtained (Figure 1a). The first peak contained the mEGF–dex conjugate and free nonreacted dextran. The second peak contained free nonreacted mEGF, and the third peak contained low molecular weight components such as free iodine and salts. It is seen that iodine is released into the low molecular fraction after the conjugation chemistry is carried out. The contents of the first two peaks were confirmed by electrophoresis (see below).

Panels a–c of Figure 1 show that the coupling efficiency increased with time up to about 24 h. Since only mEGF was radiolabeled, only the conjugate could be monitored in the first peak and not the free dextran. Results from several incubation periods are summarized in Figure 1d. The ratio of the conjugate peak radioactivity to the sum of conjugate and nonreacted mEGF peak radioactivities was calculated and defined as the conjugation efficiency. A plateau was reached after ~24 h. This reaction time was used in all conjugations. The conjugation efficiency as a function of the pH in the coupling buffer was analyzed the same way. The result is summarized in Figure 2. The maximum efficiency was found to be at around pH 8. This pH was thereafter applied in all conjugations.

A number of conjugation control experiments were then carried out. These are shown in Figure 3. In each panel a reference conjugation chromatogram showing the position of the conjugate and salt peaks is superimposed (dashed lines). Figure 3a shows the result when $NaCNBH_3$ was omitted from the reaction mixture of dextran and [^{125}I]mEGF. Only minor amounts of radioactivity could be detected at the position of the conjugate. In another experiment (Figure 3b) the dextran was omitted but $NaCNBH_3$ was added. Insignificant amounts of radioactivity were found in the conjugation peak, indicating that no aggregation of mEGF molecules takes place due to the presence of $NaCNBH_3$. A control chromatography of nonconjugate ^{125}I-labeled mEGF is shown in Figure 3c. Only a very small peak can be seen at the conjugate position.

Figure 4 shows separation of [^{125}I]mEGF–Dex conjugate and nonreacted [^{125}I]mEGF by analytical native polyacrylamide gradient gel eletrophoresis. The conjugate and mEGF are easily separated. In a separate experiment it was shown that dextran remained at the loading position. This method was used to verify the

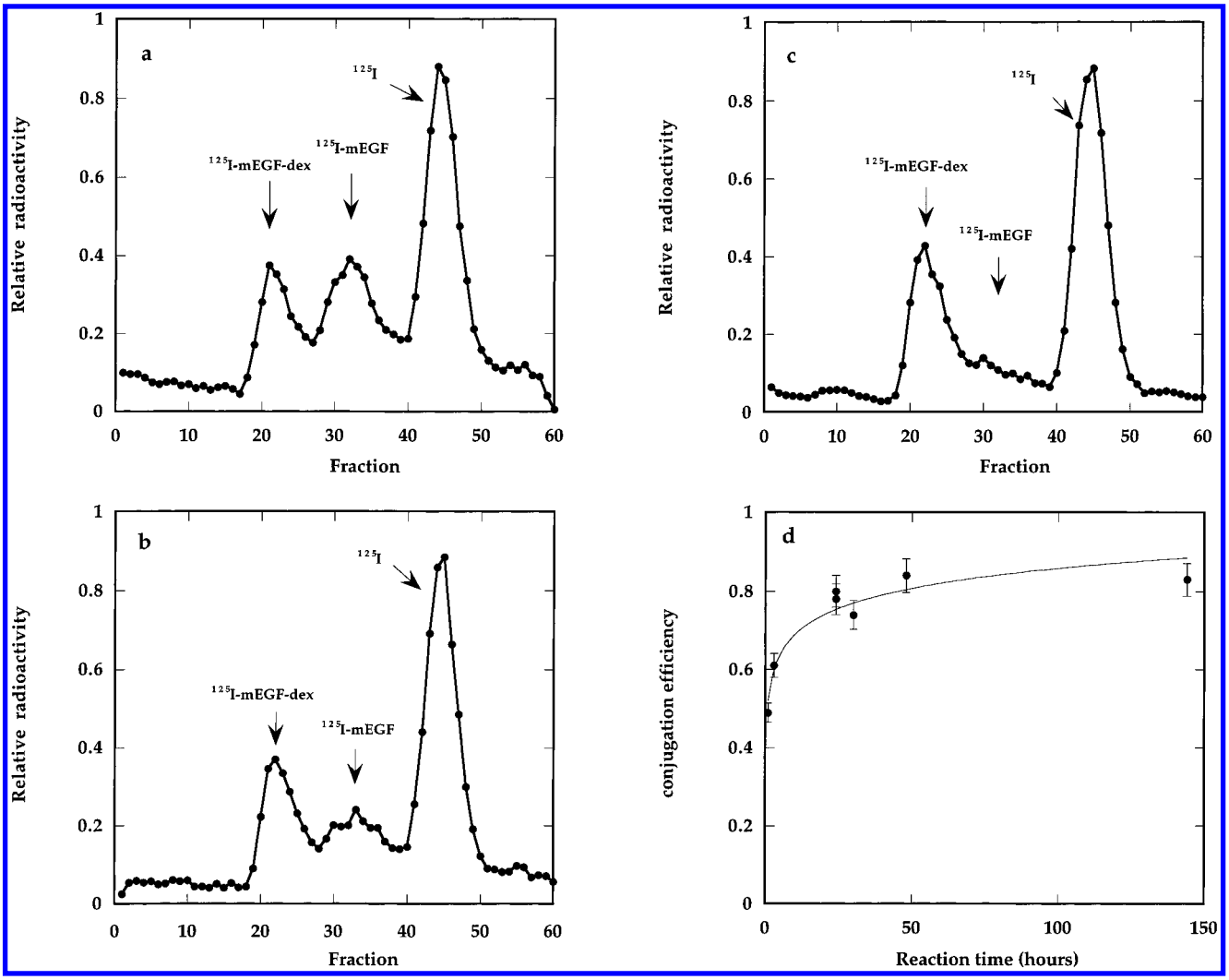

Figure 1. Gel filtration chromatogram after reductive amination of mEGF to dextran. 2.5 μg of [^{125}I]mEGF, 20 mg of dextran D14, and 8 mg of $NaCNBH_3$ were used in the reaction. The reaction was kept at room temperature (pH 8.0, 0.05 M phosphate buffer). The samples were run on a Sephadex G-50 Fine column (1 × 40 cm) with a flow rate of 0.2 mL/min. Every fraction of 0.5 mL was measured with the gamma counter (the ^{125}I channel). The results were then normalized to "relative radioactivity" so that the heights of the low molecular weight peaks (marked ^{125}I) were comparable. Panels a, b, and c show chromatograms after 1, 3, and 24 h reaction times, respectively. Panele d shows the coupling efficiency as a function of several applied reaction times. The efficiency is defined as the ratio between the conjugate radioactivity and the sum of conjugate and mEGF radioactivities. The uncertainty in each calculated point was estimated to be not >5%, which is applied as the error bars.

result of the preparative Sephadex G-50 Fine chromatography experiment.

Figure 5 shows the result of a preparative polyacrylamide electrophoresis experiment. The excess amount of noncharged dextran did not move into the 7% polyacrylamide gel but remained at the sample application position (marked B in Figure 5). It could therefore be easily removed from the conjugate solutions. The conjugate migrated more slowly than free mEGF, resulting in an almost baseline separation. Analyses on Sephadex G-50 Fine chromatography and PhastGel electrophoresis confirmed that the first peak was free mEGF and the second was the conjugate.

The result from a cellular displacement test is shown in Figure 6. The EGF-receptor-rich cultured human glioma U-343MG cells were applied. The result shows that the radiolabeled [^{125}I]mEGF−Dex conjugate specifically bound to the EGF receptors on the cell surface without nonspecific interactions since the binding could be completely inhibited by nonradioactive free mEGF.

The stability of the purified [^{125}I]mEGF−Dex conjugate was analyzed by Sephadex G-50 Fine chromatography after exposure to conditions described under Experimental Procedures. The absence of peak broadening and other changes in the chromatograms (not shown) and lack of radioactivity at the mEGF position support the assumption that the conjugate is stable at pH values of 4−9 and in 3 M NH_4SCN, 7 M urea, 1 M NaCl, 30% ethylene glycol (w/v), or serum. Binding to cultured cells was finally analyzed, and it was shown that such treatments did not inhibit the binding to the EGF receptors. Some variations in binding were seen (Figure 7), but it was only in the case of treatment with pH 9 that the binding decreased significantly in relation to the "positive control" as analyzed with a paired T-test ($p < 0.05$). However, in that case the binding was significantly higher ($p < 0.05$) than in the "negative control" cell sample given 0.5 μg/mL nonradioactive mEGF to displace the binding. Thus, the conjugate seemed reasonably stable with regard to both the conjugate linkage and the receptor binding.

DISCUSSION

Reductive amination is a nucleophilic addition reaction. The aldehyde group on the reducing end glucose of dextran provides the carbonyl group to the nucleophilic agent, i.e., the N-terminal α-amino group of mEGF. Protonization enhances the reactivity of the carbonyl

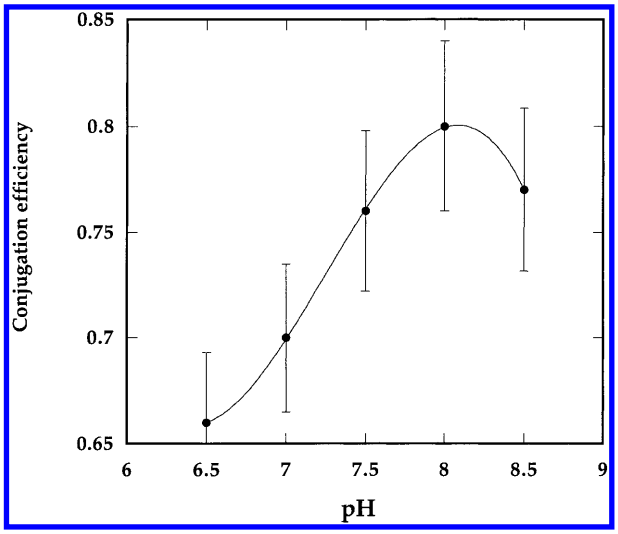

Figure 2. Reductive amination efficiency as a function of the applied pH during conjugation. All reaction times were 24 h (at room temperature). The conjugation solution was run on a Sephadex G-50 Fine (1 × 40 cm) column for gel filtration to separate conjugate and free mEGF. The quotient between the amount of radioactivity in the conjugate peak and the total radioactivity in the conjugate and free mEGF peaks was defined as the conjugation efficiency. The uncertainty in each calculated point was estimated to be not >5%, which is applied as the error bars.

group but reduces the reactivity of the N-terminal amino group of mEGF. Obviously there has to be a compromise, i.e., an optimum pH for the conjugation. The N-terminal α-amino group on a protein or a peptide has a p*K* value of ∼8. This means that most of the terminal amino groups on mEGF should be deprotonated at pH values >8. In Figure 2 the reductive amination reaction reached a maximum rate at about pH 8. At this pH the reactivity of the arginine residues in the reaction mixture is significantly reduced since their p*K* value is 12.

There are two consecutive reaction steps in reductive amination. First, the terminal amino group of mEGF reacts with the aldehyde group on the dextran end and becomes a Schiff base. However, this Schiff base is not stable, especially at acidic pH, so the reaction can reverse to produce free amino and aldehyde groups. Sodium cyanoborohydride ($NaCNBH_3$) is used to convert the Schiff base into a stable secondary amine.

The amount of the free aldehyde in glucose is, under equilibrium conditions, only ∼0.024% in a neutral solution. The situation for the end aldehyde group in dextran is probably the same. On a time scale this means that each end glucose is available in free aldehyde form only on average 0.024% of the reaction time. A consequence of this is that only trace amounts of the Schiff bases would be formed in the reaction if no sodium cyanoborohydride was present. So, when an end glucose opens, exposing a free aldehyde, this group is "captured" by the sodium cyanoborohydride mediated process, giving the stable final product, mEGF−Dex. Thus, it is not surprising that many hours are required to reach a plateau in the conjugation reaction (Figure 1d).

The mEGF−Dex conjugate and the free mEGF have mean molecular masses of 20 and 6 kDa, respectively, and can easily be separated on Sephadex G-50 Fine. However, it was not possible, in this way, to get rid of free dextran since it eluted together with the conjugate. It is essential to remove the free unconjugated dextran since it can in tumor therapeutical applications, after loading with toxic agents, possibly cause unwanted systemic toxic effects to normal tissues. Preparative

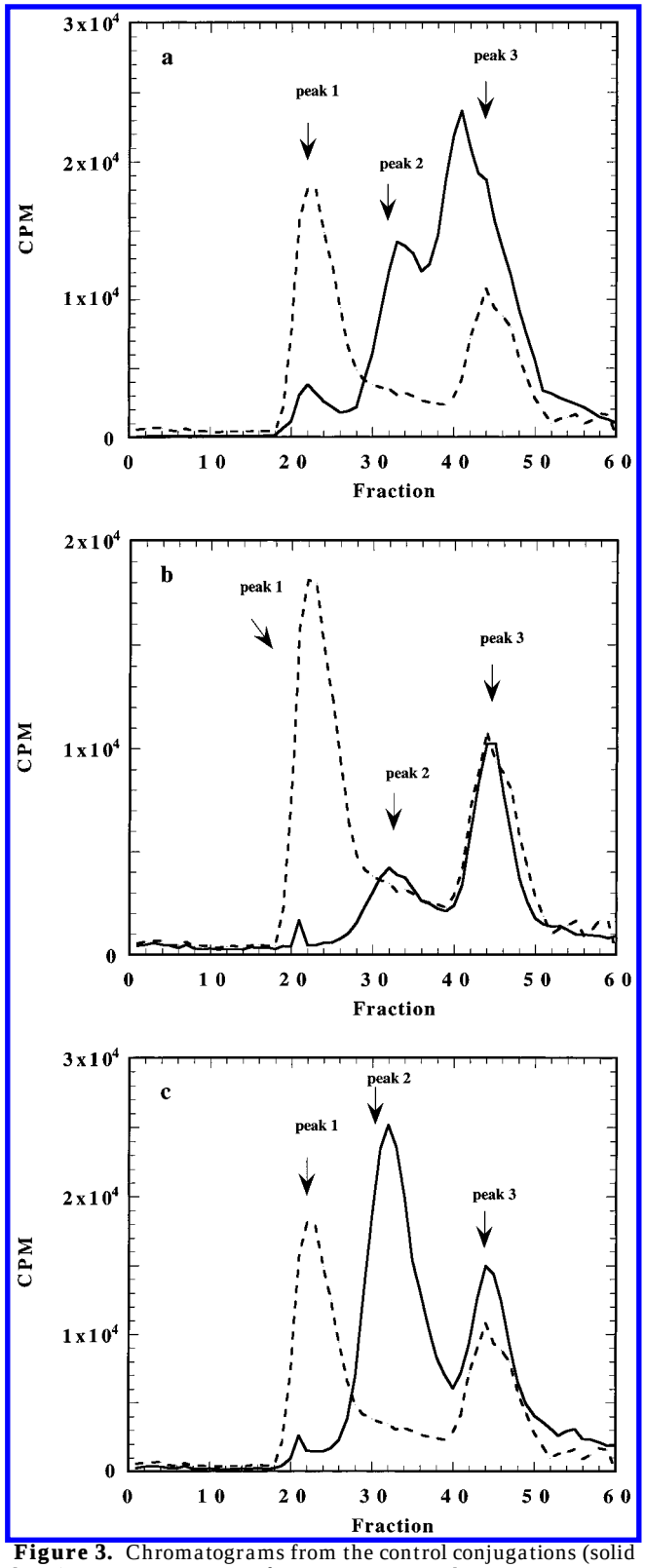

Figure 3. Chromatograms from the control conjugations (solid lines). For comparison, reference conjugate chromatograms are superimposed with dashed lines. The preparations were made with 24 h of reaction time (pH 8.0, 0.05 M phosphate buffer). The gel filtration was run on a Sephadex G-50 Fine (1 × 40 cm) column with 0.2 mL/min flow rate. Every 0.5 mL fraction collected was measured with the gamma counter using the ^{125}I channel giving cpm (counts per minute). Peaks 1, 2, and 3 represent conjugate, free mEGF, and free iodine, respectively. Panel a shows the result when [^{125}I]mEGF was incubated with dextran D14 without $NaCNBH_3$. Panel b shows the result when [^{125}I]mEGF was incubated with $NaCNBH_3$ without dextran, and panel c shows the result when [^{125}I]mEGF was run on the column without prior treatment together with $NaCNBH_3$ and dextran.

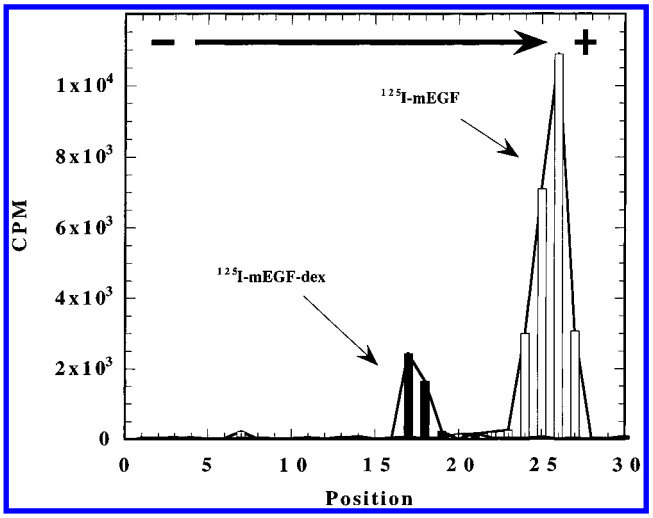

Figure 4. Native gradient polyacrylamide electrophoresis run with the Phast system. Samples of 4 μL were loaded onto a Phast Gel (8−25% gradient). The electrophoresis was run at 400 V for 30 min with native buffer. Each lane of the gel was then cut into 30 pieces and placed in 0.5 mL of PBS for 30 min. The samples were then measured with the gamma counter using the ^{125}I channel giving cpm. Minus and plus symbols represent the negative and positive electrodes in the electrophoresis, respectively. The arrow shows the migration direction in the electrophoresis. Fraction 5 was the sample loading position.

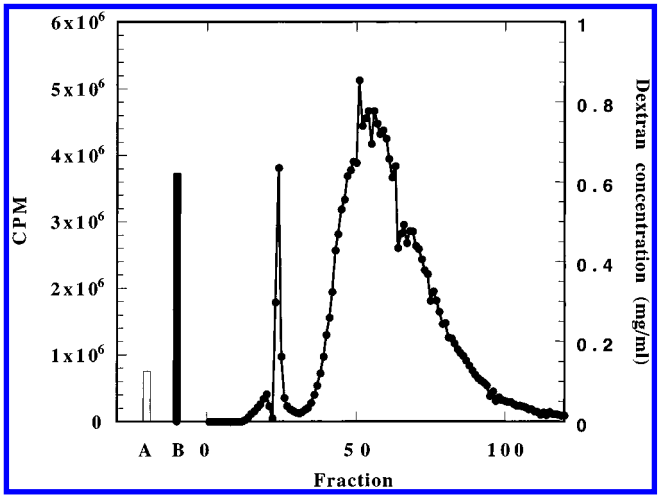

Figure 5. Preparative gel electrophoresis [polyacryamide gel = 7% T for isolation (4 cm), 4% T for stacking (1.5 cm)]. The electrophoresis was run at 300 V, 3 mA, and 1 W limit using the MiniPrep Cell equipment. A volume of 0.5 mL of conjugate sample (from the Sephadex G-50 Fine separation) was applied. The obtained 1 mL fractions were collected at 0.1 mL/min. The radioactivity was measured in the gamma counter giving cpm, and the obtained data are plotted with solid circles. The symbol A (open bar) shows the remaining radioactivity in the polyacryamide gel, and the symbol B (solid bar) shows the dextran concentration in a 1 mL buffer solution from the "upper chamber" sample application position (near the negative electrode) after the chromatography run was finished.

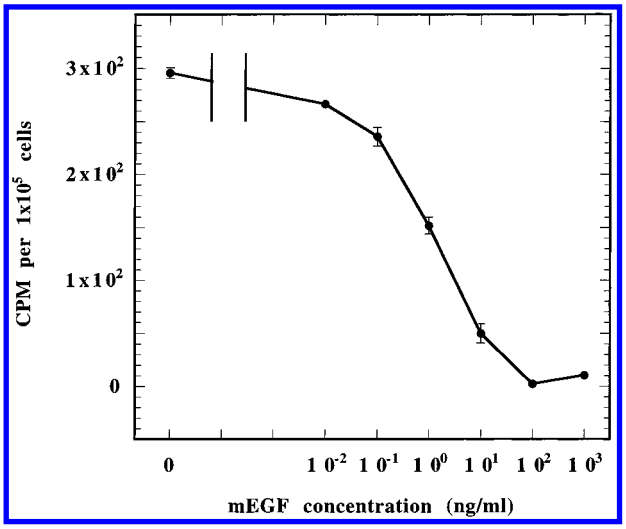

Figure 6. Displacement curve showing the decreasing [^{125}I]-mEGF−dextran conjugate binding to U-343MGaCl2:6 cells when increasing amounts of nonradioactive mEGF were added to the incubation medium (each incubation was made at 37 °C for 1.5 h). The [^{125}I]mEGF−dextran samples were taken from Sephadex G-50 Fine separations. Each point corresponds to analysis of three cultures, and mean values and maximum variations were given. The ^{125}I radioactivity was analyzed in the gamma counter giving cpm.

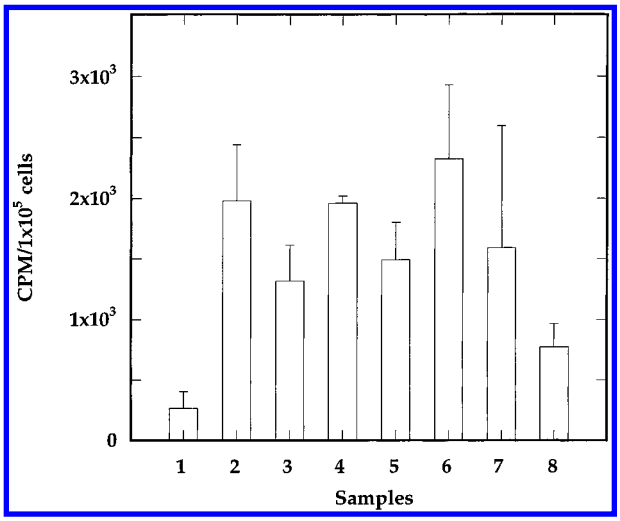

Figure 7. Conjugates were exposed to different buffers to test their stability and their EGF-receptor binding capacity. The receptor binding capacity was tested with U-343MGaCl2:6 cells (1.5 h of incubation with the cells). The histogram shows, from the left, sample 1 [binding of [^{125}I]mEGF−dextran during displacement with 0.5 μg/mL nonradioactive mEGF (negative control)], sample 2 [binding of [^{125}I]mEGF−dextran (positive control)], samples 3−8 [binding of [^{125}I]mEGF−dextran after 1 h of exposure to 3 M NH_4SCN (3), 7 M urea (4), 30% ethylene glycol (5), 1 M NaCl (6), pH 4 (7), and pH 9 (8)]. The ^{125}I radioactivity was analyzed in the gamma counter giving cpm. Mean values and maximal variations from four culture dishes are shown.

polyacrylamide gel electrophoresis was used to purify the conjugate sample from free nonreacted dextran which is noncharged and does not migrate in the electrophoresis gel. Because of the large difference in their molecular weights, mEGF and the mEGF−Dex conjugate could be separated on both analytical and preparative polyacrylamide gel electrophoresis.

The relatively small amount of free [^{125}I]mEGF (~5% of the total radioactivity in Figure 5) most likely was an effect of cross-contamination between the two neighboring peaks in the gel filtration experiment (see for example the peaks in Figure 1a). Thus, preparative electrophoresis can efficiently separate the conjugate from excess nonreacted dextran and remove mEGF impurities, resulting in a very pure mEGF−dextran preparation. Possibly the preparative gel electrophoresis method can be optimized to a "one step" purification method giving adequate separation of [^{125}I]mEGF−Dex, [^{125}I]mEGF, and nonreacted dextran.

It is seen in Figure 1 that iodine is released into the low molecular weight fraction after the conjugation chemistry is carried out. The mEGF was purified on a desalting column directly after iodination. However, the iodinated mEGF batches were then stored for different

times (up to 14 days) in relation to the dextranations and therefore free iodine appeared, due to radiolysis, in the final purification step on the Sephadex G-50 Fine column. However, even if the dextranation was made directly after the iodination, some low molecular weight iodine appeared. The reason for this is not clear, but it is not a major problem in future experiments and in the planned clinical tests since the conjugates will be purified directly before injections.

The stability of the conjugate against chemical degradation during exposure to human serum or various pH or ionic strengths and the retained ability to bind to the EGF receptors indicate that the conjugate is suitable for the planned biological experiments and clinical trials. Animal experiments, with transplanted tumors, to analyze the uptake in the tumors as well as in normal tissues are now in progress. Furthermore, we have, together with the oncologists at the university hospital in Uppsala, decided to use the described EGF−dextran for postoperative intracavitary injections (into the cavity left after the operation) in malignant glioma patients to analyze whether tumor cells seem to spread into the surrounding brain tissue. Those tests will be made on patients whose primary operation material proved to contain tumor cells with large amounts of EGF receptors. The EGF−dextran was in the present study labeled with ^{125}I but will be, in the patient studies, labeled with ^{124}I for imaging with PET (and later labeled with ^{131}I if nuclide therapy is requested).

The dextran probably protects the protein ligand against proteolysis, as has been reported in other cases when carbohydrates and proteins have been conjugated (Andersson et al., 1991; Blomhoff et al., 1983; Kato et al., 1990; Marshall, 1980; Melton et al., 1987; Olsson et al., 1994; Sjöström et al., 1997; Srivastava, 1991; Yasuda et al., 1990). In the next phase of this work cytotoxic agents will be coupled to the mEGF−Dex for which optimum conditions have to be developed. A high stability of the conjugate is then essential.

After coupling of toxic agents to the dextran part, the conjugate will be used in targeted treatment of tumors that overexpress the EGF receptor. EGF-based conjugates are known to be internalized (Andersson et al., 1991; Gedda et al., 1996; Olsson et al., 1994), and such conjugates are therefore of special interest when loaded with "short range" radionuclides. Normal cells might also have EGF receptors, but the amount of receptors per cell is dependent on the cell type and whether the cells are up or down regulated, for example, due to normal ligand exposure (Carpenter, 1987). The highest values are in the liver, as has been described in studies on mice and rats (Chabot et al., 1986; Dunn et al., 1986; Murakami et al., 1994; Soley and Hollenberg, 1987). Quantitative data for human liver seem to be lacking in the literature.

The choice of toxic agent, to be coupled to EGF−dextran, is a subject for further research. The optimal choice of the toxic agent might vary from application to application. Radioactive nuclides (such as high-energy beta or alpha emitters), borob-containing compounds, and toxins are all interesting alternatives (Carlsson et al., 1994; Ozawa et al., 1989; Siegall et al., 1990). It might also be possible to combine EGF−dextran with chelating agents that could chelate radioactive metals of tumor therapeutical interest.

The purpose of this work was to design and develop a well-defined conjugate that binds specifically to the EGF receptor and which can be the starting point for producing toxic tumor targeting agents. An interesting aspect, considering future applications, is that the present conjugates have a one to one molar ratio of dextran to EGF, enabling the loading of well-defined amounts of toxic agents to each conjugate molecule.

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