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Dextran-methylprednisolone succinate as a prodrug of methylprednisolone: in vitro immunosuppressive effects on rat blood and spleen lymphocytes

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Abstract

The in vitro immunosuppressive activity of a conjugate of methylprednisolone (MP) with dextran 70 kDa (DEX-MPS) was tested using the lymphocyte proliferation assay after stimulation of lymphocytes with concanavalin A (Con-A). Blood and spleen lymphocytes, isolated from drug-free male Sprague–Dawley rats, were used in the assay. First, the optimum concentration of Con-A for stimulation of lymphocytes was determined. The inhibition of the lymphocyte proliferation was then tested in the presence of 0.25, 0.5, 1.0, 2.5, 5.0, 10, 20, and 50 nM concentrations (MP equivalent) of DEX-MPS or free MP. The maximum stimulation of lymphocytes with Con-A was observed at mitogen concentrations of 2.5 and 10 μg/ml for the spleen and blood lymphocytes, respectively. For free MP, sigmoidal relationships were observed between the effect (% inhibition of lymphocyte proliferation) and the logarithm of MP concentration. Additionally, the maximum inhibitory effect (I_{max}) and MP concentration producing half of I_{max} (IC_{50}) were, respectively, 98% and 1.38 nM for the blood and 86% and 3.1 nM for the spleen lymphocytes. For MP conjugated to dextran, the response–log concentration curves were substantially shifted to the right with IC_{50} values of 40 and 52 nM for the blood and spleen lymphocytes, respectively. It is concluded that compared with free MP, the steroid attached to dextran has minimal immunosuppressive activity. Therefore, to be effective in vivo, DEX-MPS should release MP in the body.

Keywords: Methylprednisolone; Dextran-methylprednisolone conjugate; Immunosuppression; Lymphocyte proliferation; Splenocytes; Targeted delivery

1. Introduction

Glucocorticoids such as methylprednisolone (MP) have been used as immunosuppressants for the treatment of acute and chronic rejection in transplant patients for several years (Erken et al., 1993; Wiesner et al., 1994). However, the use of these and other immunosuppressive agents has been associated with a significant degree of toxicity (Stubbs and Morrell, 1973; Boitard and Bach, 1989). Consequently, efforts have been made to improve the effect/toxicity profiles of these drugs

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by altering their pharmacokinetics. For example, a liposomal formulation of MP targeted the drug to the spleen (Mishina et al., 1993) and was more effective than free drug in an experimental model of heart transplantation (Mishina et al., 1994). Alternatively, macromolecular prodrugs of immunosuppressive drugs may favorably alter the kinetics/dynamics of these drugs. In preliminary pharmacokinetic and stability studies (Mehvar, 1997; Mehvar et al., 2000), we have recently investigated the feasibility of the use of a conjugate of MP with dextran polymer with a molecular weight of 70 kDa (DEX-MPS). Based on the pharmacokinetics of dextran macromolecules (Mehvar et al., 1994), such a conjugate is expected to accumulate in reticuloendothelial tissues such as the liver and spleen which are major organs responsible for the immune response.

Previous studies using dextrans have shown that the conjugates of dextrans and therapeutic agents may be pharmacologically active in the conjugated from or need to release the active moiety in vivo to be effective. For example, the pharmacologic effects of mitomycin C-dextran conjugates have been attributed to the released drug (Kojima et al., 1980). However, dextra–insulin conjugate is pharmacologically active by itself (Sakamoto et al., 1977). As for the immunosuppressive drugs, a recent report (Yura et al., 1999) on the conjugate of dextran with tacrolimus suggested that the conjugate has an in vitro immunosuppressive activity almost equal to that of the free drug. Therefore, the purpose of the present study was to investigate the in vitro immunosuppressive activity of DEX-MPS, in comparison with the free drug, using blood and spleen lymphocytes obtained from rats.

2. Materials and methods

2.1. Materials

Dextran with an average molecular weight of 73 kDa, 6α-methylprednisolone (MP), RPMI 1640 (HEPES modification with 1-glutamine), concanavalin A (Con-A), Histopaque lymphocyte separation medium, penicillin/streptomycin, and 2-mercaptoethanol were obtained from Sigma Chemical (St. Louis, MO). 6α-Methylprednisolone 21-hemisuccinate (MPS) was purchased from Steraloids (Wilton, NH). [Methyl-^{3}H]-thymidine (2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other reagents were analytical grade and obtained through commercial sources.

Dextran-methylprednisolone succinate (DEX-MPS) was synthesized, purified, and characterized as described before (Mehvar, 1999). The degree of substitution of DEX-MPS was 8 mg of MP per 100 mg of the powder.

2.2. Collection of blood and spleen lymphocytes

The procedures involving animals used in this study were approved by our institutional animal committees. Adult, male Sprague–Dawley rats were purchased from a commercial source and housed in a light- and humidity controlled animal facility at least 2 days prior to the experiments. The animals had free access to water and food. The animals were euthanized by means of carbon dioxide, and blood was collected into heparinized syringes by cardiac puncture. Additionally, the spleen was collected. The spleen was pushed gently through a stainless steel mesh screen, and the cells were suspended in an RPMI solution in a sterile petri dish ($\sim$ 50 ml). Blood (3 ml) was also diluted with RPMI (5 ml). Aliquots (8 ml) of the blood or spleen cell suspensions were then layered on top of a Histopaque solution (3 ml) in a sterile conical tube. After centrifugation (400 g for 30 min), the lymphocyte band was taken and washed with RPMI via an additional centrifugation. The final cell pellet was suspended in RPMI complete medium, containing penicillin (100 IU/ml), streptomycin (100 μg/ml), heat-inactivated rat serum (2.5%), and 2-mercaptoethanol (5×10^{-5} M). The cells were then counted using a hemocytometer.

2.3. Lymphocyte proliferation assay

The in vitro immunosuppressive activity of MP or DEX-MPS was determined using the spleen

and blood lymphocyte proliferation assay (Yaqoob et al., 1994). Briefly, to each well in a 96-well microplate were added 100 μl of the cell suspension and 100 μl of an RPMI complete solution containing Con-A and MP or DEX-MPS to make a final cell count of 2×10^5 cells/well. The final concentrations of MP or DEX-MPS in the wells were 0, 0.25, 0.5, 1, 2.5, 5, 10, 20, or 50 nM (MP equivalent). Control wells were prepared similarly without Con-A or drugs. The plates were then incubated at 37°C in a humidified incubator containing air: CO_2 (95:5). After 48 h of incubation, 2 μCi of tritiated thymidine was added to each well, and the plate was incubated for an additional 18 h. Finally, the cells were harvested on a glass fiber paper using a Skatron Cell Harvester (Sterling, VA), and the papers were soaked in 3 ml of a Cytoscint liquid (ICN, Costa Mesa, CA) for counting on a Beckman LS 1800 counter. All samples were prepared in triplicates.

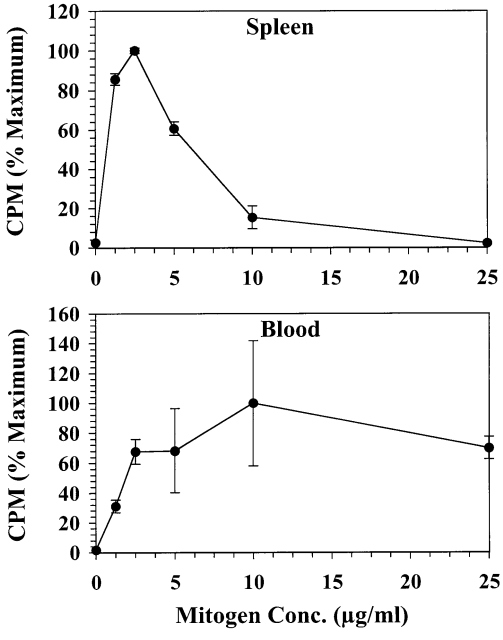

Fig. 1. The effects of mitogen (concanavalin A) concentration on the spleen (top) and blood (bottom) lymphocyte proliferation assay. The count per min (CPM) is directly related to the uptake of radioactive thymidine by the lymphocytes ($n = 3$). Bars indicate standard deviations.

To determine the optimum concentration of mitogen (Con-A) at which maximum proliferation of lymphocytes is observed, the proliferation assay was first conducted in the presence of 0, 1.25, 2.5, 5, 10, and 25 μg/ml Con-A for both blood and spleen lymphocytes. The actual experiments were then conducted in the presence of Con-A concentration of 2.5 or 10 μg/ml for the spleen and blood lymphocytes, respectively.

The viability of the cells was $> 90\%$, as determined microscopically by the trypan blue exclusion test.

2.4. Analysis of data

The relationship between the inhibitory effects of MP or DEX-MPS on the lymphocyte proliferation was studied using the sigmoidal E_{max} model as shown in the following equation

$$I = \frac{I_{max} C^n}{IC_{50}^n + C^n},$$

where I, I_{max}, C, and IC_{50} are percentage of inhibition of lymphocyte proliferation compared with baseline, maximum inhibition, concentration of MP or DEX-MPS in the media, and the drug concentration resulting in half of maximum inhibition, respectively. Additionally, the exponent n determines the steepness of the curve. The parameters I_{max}, IC_{50}, and n were estimated by using nonlinear regression analysis of the data.

3. Results

The proliferation profiles of blood and spleen lymphocytes in the presence of mitogen (Con-A) concentrations in the range of 0–25 μg/ml are depicted in Fig. 1. Based on these data (Fig. 1), the responses of the spleen and blood lymphocytes to the mitogen are maximal at mitogen concentration of 2.5 and 10 μg/ml, respectively. Therefore, these mitogen concentrations were used in all the subsequent studies in the presence of MP or DEX-MPS.

The in vitro effects of MP and DEX-MPS on the spleen and blood lymphocyte proliferation are demonstrated in Fig. 2 for both the free and

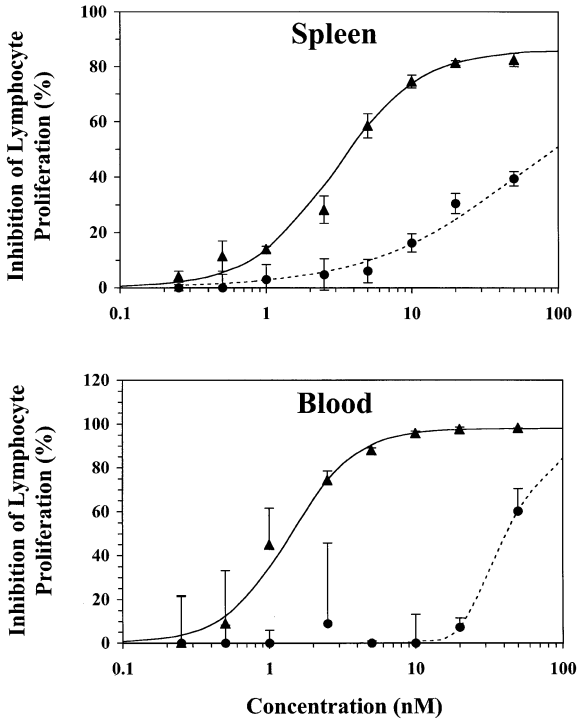

Fig. 2. The effects of free (▲) or dextran-conjugated (●) MP concentrations on the inhibition of spleen (top) and blood (bottom) lymphocyte proliferation ($n = 3$). Bars indicate standard deviations.

Table 1
Pharmacodynamic parameters of MP and DEX-MPS for inhibition of spleen and blood lymphocytes of rats

Parameter[a]	Spleen		Blood	
	MP	DEX-MPS	MP	DEX-MPS
IC_{50}, nM	3.03	52.1	1.38	39.8
I_{max}, %	86.3	80.2	98.1	87.6
n	1.49	0.84	1.90	3.5

[a] IC_{50}: concentration producing half of maximum inhibition; I_{max}: maximum inhibition; n: steepness coefficient

conjugated MP. For spleen lymphocytes (Fig. 2, top), the relationship between the inhibition of lymphocyte proliferation and the concentration of free MP was a classical sigmoidal curve with an effect plateau reaching at MP concentrations between 10–50 nM. For DEX-MPS in the spleen, the curve was substantially shifted to the right and a plateau was not observed within the studied concentration range (0.25–50 nM, MP equivalent). Qualitatively similar profiles were observed for the blood lymphocytes (Fig. 2, bottom).

The pharmacodynamic parameters of the free and conjugated MP are reported in Table 1. For free MP, the maximum inhibition of the blood lymphocyte proliferation (98%) appeared to be slightly higher than that for the splenocytes (86%). Additionally, the affinity of free MP towards blood lymphocytes (IC_{50} of 1.38 nM) was apparently higher than that for the splenocytes (IC_{50} of 3.03 nM). Although the estimated maximum inhibition values of DEX-MPS were relatively close to those of free MP, there were substantial differences between MP and DEX-MPS in their IC_{50} values (Table 1); the IC_{50} values of DEX-MPS for the spleen and blood lymphocytes were, respectively, 17- and 28-fold of those for free MP (Table 1).

4. Discussion

Lymphocyte proliferation assay is a well-accepted method for determination of the immunomodulating effects of corticosteroids such as MP (Mishina and Jusko, 1994). We used this method to compare the in vitro effects of free MP with those of the steroid covalently bound to dextran macromolecule. The data using both blood and spleen lymphocytes (Fig. 2 and Table 1) clearly indicate that at equivalent concentrations, DEX-MPS is substantially less effective than free MP.

Theoretically, the low immunomodulating effect of DEX-MPS, observed in our in vitro study (Table 1 and Fig. 2), may be due to a direct effect of the conjugate and/or a partial release of free MP from DEX-MPS during the incubation period (66 h). Our previous studies (Mehvar et al., 2000) have shown that at a physiologic pH of 7.4, MP is formed from DEX-MPS with an apparent rate constant of 0.014 h^{-1} (half life of ~ 50 h). How-

ever, as the pH decreases to below 7, the rate of hydrolysis drastically decreases, resulting in a > 20 decrease in the hydrolysis rate constant of DEX-MPS when the pH is changed from 7–5 (McLeod et al., 1993). The lymphocyte proliferation assay starts with the incubation of cells at a pH of 7.4. However, as cells proliferate during the incubation, the pH of the media becomes progressively acidic (Freshney, 1987). Therefore, it is likely that a small fraction of DEX-MPS is hydrolyzed during the incubation period, resulting in the low immunosuppressive activity observed in the presence of DEX-MPS. This postulate is consistent with similar I_{max} and higher IC_{50} values for DEX-MPS, as compared with the corresponding values for free MP (Table 1).

We attempted to quantitate the concentrations of hydrolyzed MP in the wells by HPLC after the incubation of the spleen and blood lymphocytes in the presence of DEX-MPS. However, the concentrations of MP were below the detection limits of the available HPLC assays (> 5 ng/ml). This is not unexpected as the highest concentration of DEX-MPS used in this study was 50 nM or 19 ng/ml of which only a small fraction is expected to be in the free form. Nonetheless, the observed immunomodulating effects of DEX-MPS at high in vitro concentrations (Fig. 2) are likely attributed to the free MP released during the incubation period and not due to the direct effects of the conjugate.

The significant differences between the in vitro IC_{50} values of free and dextran-conjugated MP observed in our study (Table 1) is in contrast to the recent data on a conjugate of dextran with tacrolimus reported by Yura et al. (Yura et al., 1999). These authors reported comparable IC_{50} values for the free and dextran conjugated tacrolimus using lymphocytes isolated from the rat mesenteric lymph nodes. This is interesting as the release of free tacrolimus from the conjugate, under the proliferation condition (pH 7.4 and 37°C), was very slow with a half life of 150 h (Yura et al., 1999), suggesting that the conjugate by itself is almost as effective as the free drug. The discrepancy between our results with DEX-MPS and those of Yura et al. with tacrolimus-dextran (Yura et al., 1999) may be due to methodological differences between the two studies. For instance, we used a neutral dextran in our study, whereas Yura et al. used a negatively charged dextran (carboxy-pentyl dextran) for conjugation. The electric charge may affect the release pattern of the free drug and/or the interaction of the conjugate with the receptor, if any. Additionally, although succinic acid was used in both studies as a linkage between the drugs and dextran, the tacrolimus–dextran linkage also contained a carboxy-pentyl and an *N*-hydroxysuccinamide group in the spacer arm (Yura et al., 1999). The longer spacer arm for the tacrolimus–dextran conjugate, compared with DEX-MPS, may also have contributed to the different in vitro effects of the conjugates. Nevertheless, the mechanisms of the in vitro effects of tacrolimus–dextran remains to be determined (Yura et al., 1999).

In our studies, the blood and spleen lymphocytes showed different response patterns to various concentrations of the mitogen (Fig. 1). Others (Yaqoob et al., 1994) have also shown that lymphocytes obtained from different tissues may respond differently to various stimuli or inhibitors. Additionally, it should be mentioned here that the rat serum added to our culture media for the spleen lymphocyte assay was from a different serum pool than that used for the blood lymphocyte assays. The different sources of rat serum may also have contributed to the different responses of the spleen and blood lymphocytes to the mitogen (Fig. 1).

In conclusion, the inhibitory effects of MP attached to a dextran macromolecule on blood and spleen lymphocyte proliferation was studied in an in vitro study. Compared with free MP, the steroid attached to dextran showed minimal immunosuppressive activity. Therefore, to be effective in vivo, DEX-MPS should release MP in the body.

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