Basic study on flurbiprofen protein binding inhibition by an albumin site II binding inhibitor, 6-methoxy-2-naphthyl acetic acid[a)]

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Abstract

The protein binding rate of a nonsteroidal antiinflammatory drug (NSAID), flurbiprofen (FP), is about 99%, indicating its low tissue transfer. 6-Methoxy-2-naphthyl acetic acid (6MNA) is an active metabolite of an NSAID, nabumetone (Relifen ®). These drugs strongly bind to human serum albumin (HSA) site II, suggesting that protein binding inhibition by 6MNA may promote tissue transfer of FP. We investigated cell membrane permeability of FP in the presence of 6MNA using swine kidney cell line LLC-PK1, in which the upper and lower sides of the cell membrane plate (Transwell ®) were regarded as the tissue and vascular sides, respectively. After adding 6MNA to HSA solution containing FP on the lower side, the FP levels in the upper and lower solutions and cells were measured. FP transfer to the upper side was significantly promoted by the addition of 6MNA with regard to the total and free FP levels. No change in the FP level was noted in cells or wells. It was suggested that concomitant nabumetone may promote transfer of site II binding drugs, such as FP, to the target tissue, increasing the effect even at a low dose.

Key words : human serum albumin, protein binding, site II, flurbiprofen, 6-methoxy-2-naphthyl acetic acid

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INTRODUCTION

The serum protein binding rate of a nonsteroidal antiinflammatory drug (NSAID), flurbiprofen (FP), is very high (about 99%), indicating its low tissue transfer[1)]. 6-Methoxy-2-naphtyl acetic acid (6MNA) is an active metabolite of an NSAID, nabumetone (Relifen ®). Its biological half-life is long, and the therapeutic concentration is high. These drugs strongly bind to site II of human serum albumin (HSA)[2)]. Thus, we considered that protein binding inhibition by 6MNA may promote tissue transfer of FP and increase its effect at a low dose. We previously measured the drug binding ability to binding sites on HSA and α 1-acidic glycoprotein (AGP) molecules and laboratory test values affecting the binding, and established a simple diagnostic method of pharmacological distribution to determine the timing of drug administration and increase the pharmacological effect[3-6)]. We also reported that protein binding inhibition by a free fatty acid (FFA), oleic acid (Ole), elevated the blood FP level[2)]. However, we have not investigated cell membrane permeability involved in tissue transfer of the drug, and only reported the protein binding-inhibitory phenomenon and pharmacokinetic changes in the body. We previously performed a basic study on cell membrane permeability of probe drugs in the presence of protein binding inhibitors using cells cultured on plates for cell permeation, and established this method[7)].

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Thus, using swine kidney cell line LLC-PK1, we investigated cell membrane permeability of FP in the presence of 6MNA.

MATERIALS AND METHODS

1. Preparation of LLC-PK1 cell membrane plates[7]

Commercial swine kidney-derived LLC-PK1 cells were seeded in a culture flask (25 cm^2) containing cell growth medium (Earle's Medium 199, GIBCO) supplemented with antibiotics (penicillin-streptomycin, GIBCO) and fetal bovine serum (FBS, GIBCO). The cells were cultured in a CO_2 incubator (5% CO_2-95% air, 37℃). The medium was changed following day, and the cells were further cultured for 5-6 days with changing medium and culture flask (75 or 150 cm^2) every 2 days. After culture, the cells were detached and suspended with Trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA, GIBCO), seeded in 6-well plates for cell permeation (membrane pore size: 3.0 μm, Transwell®, CORNING) at 2 x 10^6 cells per well, and cultured for 3 days. The upper and lower sides of the cell membrane plate were regarded as the tissue and vascular sides, respectively. Earle's M199 Solution (GIBCO) was used for culture and Earle's Balanced Salt Solution (EBSS, GIBCO) for setting of the HSA concentration and cell membrane permeation experiment of FP. LLC-PK1 cells were passaged 15-18 times before the experiment. The cell membrane permeation experiment of FP was triplicate or quadruplicate (n=3-4).

2. FP measurement method[2]

FP was measured using high-performance liquid chromatography (HPLC). All samples excluding free FP solution were deproteinized with methanol before injection into HPLC. The HPLC measurement conditions for FP analysis were as follows:

HPLC, SHIMADZU CLASS-VP; column, LiChrospher® RP-18 Select B (5 μm), 250 mm L. x 4.0 mm I.D.; mobile phase, 0.1 M citric acid - 0.1 M sodium acetate (pH 4.5): acetonitrile=1:1; flow rate, 1.0 mL/min; column temperature, 40℃; injection volume, 30 μL; excitation, 248 nm; and emission, 320 nm.

3. Setting of HSA concentration

The culture medium was changed to EBSS on experiment day (1.5 and 2.6 mL on the upper and lower sides, respectively). FP (final concentration: l40 μM) was added to HSA solution at various concentrations (final concentration: l0, 40, 100, 200, 400, and 600 μM), and the total and free FP levels on the upper and lower sides were measured every hour for 4 hours (free FP was measured only at 4 hours). To measure free FP, the upper and lower solutions were centrifuged at 3,000 rpm for 10 minutes and ultrafiltrated (TOSO) to prepare free FP solutions. The protein binding rate of FP was calculated from the total and free FP levels.

4. Cell membrane permeation experiment of FP

The culture medium was changed to EBSS on experiment day (1.5 and 2.6 mL on the upper and lower sides, respectively). 6MNA solution (final concentration: 50 and 100 μM) was added to the HSA and FP solutions at the final concentrations established on setting the HSA concentration: 200 μM and 40 μM, respectively, and the total and free FP levels in the upper and lower solutions were measured after 2 and 4 hours (free FP was measured only at 4 hours). In addition, the intracellular FP level was measured after the experiment. To measure intracellular FP, the membrane was detached from the plate and washed twice with EBSS in a microtube, EBSS was removed by sucking, and cells were dissolved with an equal volume of trypsin (10 x) and 2N NaOH, followed by centrifugation at 3,000 rpm for 10 minutes. The supernatant was then neutralized with an equal volume of 1N HCl and subjected to FP measurement.

5. Statistical analysis

All values were presented as the means±standard deviation. For analysis of significance of differences in the FP levels in the upper and lower solutions, analysis of covariance was employed, and Fisher's PLSD was used for multiple comparison. For analysis of significance of differences in the free and intracellular FP levels, non-repeated measures ANOVA was employed, and Dunnett's test was used

for multiple comparison. $p<0.05$ was regarded as significant.

RESULTS

1. Setting of HSA concentration

To evaluate cell membrane permeability of FP in the presence of 6MNA, the HSA concentration was established (0, 40, 100, 200, 400, and 600 μM). The FP levels in the upper solution between 0 and 4 hours are shown in Fig. 1. The HSA addition markedly reduced the level. The FP levels in the lower solution at 4 hours are shown in Fig. 2. The HSA addition markedly increased the level. The protein binding rates of FP at 4 hours in the lower solution are shown in Table 1. The protein binding rate rose as the HSA concentration increased.

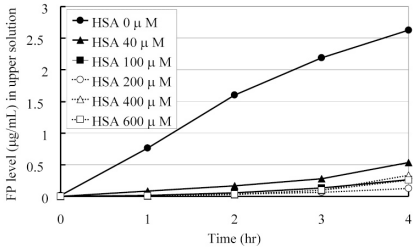

Fig. 1 The FP level (μg/mL) in the upper solution with changes in the HSA concentration

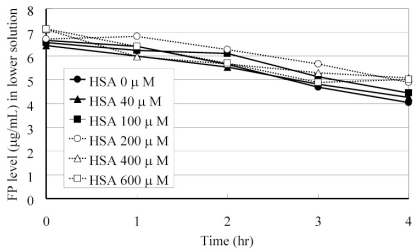

Fig. 2 The FP level (μg/mL) in the lower solution with changes in the HSA concentration

Table 1 The protein binding rate (%) of FP in the lower solution with changes in the HSA concentration

HSA concentration, μ M	40	100	200	400	600
Protein binding, %	94.7	99.4	99.9	99.9	99.9

Table 1 The protein binding rate (%) of FP in the lower solution with changes in the HSA concentration
The values are presented as the means± standard error.

2. Cell membrane permeation experiment of FP

The total FP levels in the upper solution at 0-4 hours are shown in Fig. 3. The total FP level in the upper solution was significantly increased at 4 hours in the presence of 50 μM 6MNA ($p<0.05$), but the total level in the presence of 100 μM 6MNA was lower than that in presence of 50 μM 6MNA. The total FP levels in the lower solution at 0-4 hours are shown in Fig. 4. The total level in the lower solution was not changed at 2 or 4 hours. The free FP levels at 4 hours are shown in Fig. 5. The free FP level in the upper solution was significantly elevated by the addition of 100 μM 6MNA at 4 hours ($p<0.01$). No significant change was noted in the free FP level in the lower solution or in the intracellular level at 4 hours (Fig. 6).

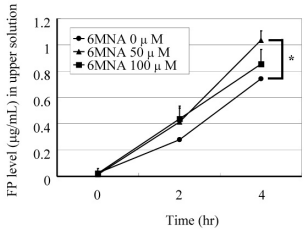

Fig. 3 The FP level (μg/mL) in the upper solution in the presence of the inhibitor
The values are presented as the means± standard error (n=4).
*; $p<0.05$

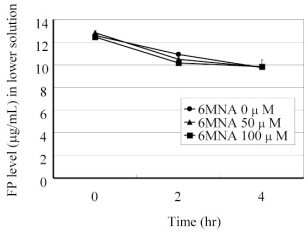

Fig. 4 The FP level (μg/mL) in the lower solution in the presence of the inhibitor
The values are presented as the means± standard error (n=4)

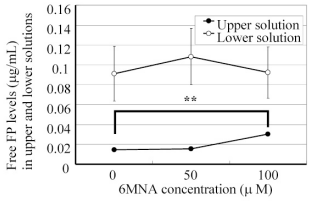

Fig. 5 The free FP levels (μg/mL) in the upper and lower solutions in the presence of the inhibitor
The values are presented as the means±standard error (n=4). **; $p<0.01$.

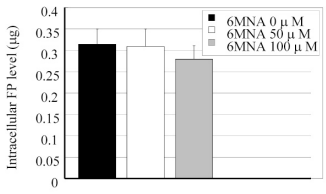

Fig. 6 The intracellular FP level (μg) in the presence of the inhibitor
The values are presented as the means± standard error (n=4).

DISCUSSION

Cell membrane permeability of FP in the presence of 6MNA was investigated using swine kidney cell line LLC-PK1. The addition of 6MNA significantly elevated the total and free FP levels.

Since the blood HSA level is about 600 μM, the cell membrane permeability experiment of FP should be investigated at an HSA concentration of 600 μM. However, HSA is a useful human-derived reagent. Thus, we investigated the HSA concentration at which the protein binding rate of FP can be maintained at 99%, and performed the experiment at the minimum HSA concentration. A high protein binding rate of FP was confirmed, as reported by Tohno et al.[1], and the protein binding rate of FP was 99.9% at HSA concentrations of 200, 400, and 600 μM. Based on these, the HSA concentration was set at 200 μM in this experiment.

Cell membrane permeability of FP in the presence of 6MNA was investigated using LLC-PK1 cells. The 6MNA addition significantly elevated the total and free FP levels, showing that cell membrane permeability of FP was increased. 6MNA may have prevented FP from protein binding by binding to HSA site II, which elevated the free FP level in the lower solution and promoted their transfer to the upper solution.

The total FP level in the upper solution at 4 hours in the presence of 100 μM 6MNA was lower than that in the presence of 50 μM 6MNA as shown in Fig. 3. We considered its reason as follows: The total FP level in the upper solution in the presence of 100 μM 6MNA tended to be higher than that in the presence of 50 μM 6MNA at 2 hours as shown in Fig. 3, and the free FP level in the upper solution was significantly higher than that in the presence of 50 μM 6MNA at 4 hours as shown in Fig. 5. The total FP level in the upper solution at 4 hours should have been higher in the presence of 100 μM 6MNA, but free FP may have transferred to the lower solution due to its increased level, reducing the total FP level in the upper solution. However, no significant changes

were noted in the total or free FP level in the lower solution at 4 hours as shown in Figs. 4 and 5, respectively.

Kawai et al. recently reported that scintigraphy with a radioactive imaging diagnostic agent, ^{123}I-N-isopropyl-p-iodoamphetamine (IMP), could be performed at a low dose using 6MNA in monkeys[8]. Both IMP and 6MNA bind to albumin site II. They utilized the increase in the free IMP level by prior binding of 6MNA to albumin site II. Their finding may have been due to increased cell membrane permeability demonstrated in our study: inhibition of binding to albumin in blood vessels may have promoted tissue transfer of IMP.

6MNA increased cell membrane permeability of FP by inhibiting protein binding of FP, suggesting that concomitant nabumetone (prodrug of 6MNA, Relifen®) enhances transfer of site II-binding drugs, such as FP, to the target tissue, increasing the effect at a low dose. The series of our findings suggest that adequate use of safe protein binding inhibitors may promote tissue transfer. We are planning to develop a dose reduction method for high-risk drugs based on these basic studies and evidence obtained from various human cells.

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アルブミンサイトⅡ結合阻害薬である6-メトキシ-2-ナフチル酢酸によるフルルビプロフェンの蛋白結合阻害に関する基礎的検討[a)]

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日本語要旨

非ステロイド性抗炎症薬（NSAID）フルルビプロフェン（FP）の蛋白結合率は約99%であり、組織移行性が低い。6-メトキシ-2-ナフチル酢酸（6MNA）は、NSAIDの1つであるナブメトンの活性代謝産物である。これらの薬物は、ともにヒト血清アルブミン（HSA)のサイトⅡに強く結合する。私たちは、LLC-PK1を用いて6MNA添加時のFPの細胞膜透過性について調べた。この実験では、細胞膜プレートの上側を組織側、下側を血管側と想定した。下側のFPを含むHSA溶液に6MNAを添加後、上側、下側のFP濃度を測定した。上側へのFPの移行量は6MNAの添加により、Total FPおよびFree FP濃度ともに有意な増加を示した。下側の量は変化が見られなかった。臨床におけるナブメトンの併用はFPのようなサイトⅡ結合性薬物の標的組織への移行量を増加させ、少量においても効果を高める可能性が示唆された。

キーワード：ヒト血清アルブミン, 蛋白結合, サイトⅡ, フルルビプロフェン, 6-メトキシ-2-ナフチル酢酸