A novel phenoxazine derivative suppresses surface IgM expression in DT40 b cell line

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【 学位論文題目 】

A Novel Phenoxazine Derivative Suppresses Surface IgM Expression in DT40 B Cell Line

(Phenoxazine 化合物 Phx は B 細胞において特異的なIgM 産生抑制効果を持つ)

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INTRODUCTION

Actinomycin D is known to inhibit the activity of DNA-dependent RNA polymerase to intercalate DNA, and has strong anti-tumour activity. Tomoda *et al.* found that 2-amino-4, 4α-dihydro-4α, 7-dimethyl-3H-phenoxazine-3-one (Phx) was produced *in vitro* by the reactions of 2-amino-5-methylphenol with human or bovine hemoglobin. Phx is a novel phenoxazine derivative and has the three rings structure, in commom with actinomycin D as phenoxazinone. In contrast, Phx is soluble in water(1.2mM at 37°C), and exerts anti-tumour effects *in vitro* on a variety of cultured human carcinoma cell lines. Moreover, it has been shown that administration of Phx to mice transplanted with Meth A carcinoma cells or leukemia cells caused extensive suppression of the growth of transplants. Unlike Actinomycin D, Phx has no DNA intercalating activity. Phx can be expected to become available for the treatment of cancer in the future; however, the mechanism of Phx has remained unclear.

In this study, we have investigated the effect of Phx on BCR signalling and immunoglobulin expression. Phx markedly reduced the IgM expression and secretion of IgM in B cells, resulting in the inhibition of BCR-mediated signaling. It is expected that Phx may become a therapeutic agent that normalizes the abnormal enhancement of immunoglobulin production.

MATERIALS AND METHODS

The Cy3-conjugated anti-goat IgG and FITC-conjugated anti-mouse IgG secondary antibodies were purchased from Sigma. The monoclonal antibody M4, an anti-chicken IgM, was used for stimulation of BCR in DT40 cells. Phx was synthesized and purified as described previously (Tomoda *et al.*, 2001). Anti-phosphotyrosine (4G10) and anti-Shc antibodies were obtained from Upstate Biotechnology. Immunoblotting and confocal microscopy of IgM were performed using the goat anti-chicken IgM-μ chain specific antibody. Anti-Syk polyclonal antibodies (N-19 and C-20) were from Santa Cruz Biotechnology, Inc. Anti-α-tubulin antibody was from Amersham. Anti-trans-Golgi Network38 antibody was from Oncogene Research Products. MG-132 was purchased from Calbiochem.

Cell culture-DT40 chicken B cells were kindly provided by Dr. Kurosaki (Kansai Medical University, Moriguchi, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10 % FCS, 2 mM glutamine, 100 U /ml penicillin and 100 mg/ml kanamycin in a humidified 5 % CO2 atmosphere.

Determination of cell proliferation and cell survival- Cells were seeded at 10^6 cells/ml and were cultured for 24 h in the presence of various concentrations of Phx. Cell numbers were determined using a Coulter counter. Cell viabilitywas confirmed by trypan blue dye exclusion method.

Immunoprecipitation and immunoblotting- Cells (10^7cells/ml) were collected and washed once. Stimulated cells were solubilized in ice-cold lysis buffer. Cell Lysates were clarified by centrifugation at 12,000 x g for 10 min at 4°C. The supernatants were incubated sequentially (1 h for each incubation) with antibodies and proteinA-Sepharose 4FF (Sigma) at 4°C, and immunoprecipitates were separated by SDS-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes. Blots were probed with the indicated antibodies and immunoreactive proteins were visualized by the Enhanced Chemiluminescence detection system.

Immunofluorescence study-Cells (10^6 cells/ml) were treated with or without 50 μM Phx for 12 h. Cells were attached to coverslips pre-coated with poly-L-lysine (100 μg/ml) for 10 min at room temperature. Adherent cells were stimulated with or without M4 (4 μg/ml) in regular culture medium for 5 min at 37°C. Cells were fixed with 4 % paraformaldehyde and stained with goat anti-chicken IgM plus Cy3-conjugated anti-goat IgG antibodies. Double immunofluorescence stainings were performed using mouse anti-TGN38 and FITC-conjugated anti-mouse IgG antibodies. Cells undergoing staining of intracellular proteins were permeabilized with 0.1 % Triton X-100 and then immunostained. Laser scanning confocal micriscopy was performed using a Bio-Rad MRC-1024 confocal system.

RESULTS

Phx inhibits the proliferation of DT40 B cells-We first examined the effect of Phx on the proliferation of DT40 chicken B cells. DT40 cells were maintained in their normal medium with various doses of Phx. The increase in cell number was almost identical at all dose levels during the first 12 h of treatment. However, exposure to Phx for an extra 12 h (24 h total) resulted in a dose-dependent inhibition of cell proliferation. Treatment with 50μM Phx for 12 h kept the percentage of nonviable cells at less than 2%, the same as controls. Even in the presence of 100μM Phx more than 95% of the cells were viable as determined by trypan blue dye exclusion.

Treatment of B cells with Phx inhibits BCR signaling and reduces the expression of IgM-It is clear that the BCR plays a central role in determining the fate of B cells. We studied the effect of Phx on intracellular signal transduction following BCR stimulation. One of the earliest events following BCR stimulation is the induction of tyrosine phosphorylation of cellular proteins. DT40 cells pretreated with 50 μM Phx for a short period (30 min)

were stimulated by an anti-IgM antibody(M4), and then the cell lysates were analysed by immunoblotting with anti-phosphotyrosine antibody. Pretreatment with Phx for 30 min had no effect on tyrosine phosphorylation of cellular proteins upon BCR stimulation. On the other hand, pre-treatment with 100mM Phx for 12 h inhibited tyrosine phosphorylation of cellular proteins in both unstimulated (-M4) and BCR-stimulated (+M4) cells. In DT40 cells, total tyrosine phoshporylation requires activated Syk, which is downstream from BCR. The tyrosine phosphorylation of Syk and Shc following BCR stimulation were inhibited by treatment with Phx for 12 h.

We further examined whether treatment with Phx for 12 h had an effect on the expression of signalling molecules. BCR is composed of heavy chains Igμ and light chains. Anti-IgM immunoblotting of the same membrane clearly showed reduced IgM expression in B cells pretreated with Phx for 12 h. However, the expression of Syk and α-tubulin were not affected by Phx pretreatment of B cells. These results suggest that treatment with Phx for 12 h inhibits BCR expression resulting in reduced tyrosine phosphorylation of cellular proteins following BCR stimulation.

The basic structure of Phx is similar to that of Actinomycin D, which is known to intercalate DNA and inhibit RNA synthesis. We compared the effect of Phx with that of Actinomycin D on IgM expression. IgM expression after treatment of B cells with Phx started to decreased at 4 h and drastically reduced by 12 h. The treatment with Phx for 12 h has no effect on the expression of Syk or α-tubulin. On the other hand, the effect of Actinomycin D on the reduction of IgM expression appeared more slowly than that of Phx. The decrease in IgM expression by Actinomycin D was small at 16 h and significant at 32 h. Down-regulation of Syk protein expression followed a similar time course as IgM. We carried out the same experiment using cycloheximide, an inhibitor of protein synthesis, and obtained similar results. Thus, unlike actinomycin D and cycloheximide, Phx rapidly inhibited the expression of IgM but not Syk, suggesting that Phx has a mode of action distinct from both of these reagents.

Treatment with Phx reduces expression of IgM both at cell surface and in inside the cell- B cells produce and store immunoglobulin in intracellular organelles. To examine the effect of Phx on the expression of BCR at the cell surface we performed confocal microscopic studies. B cells were stained with anti-IgM and Cy3-conjugated secondary antibodies to allow visualization of the surface IgM component of the BCR. At basal stage, IgM was uniformly distributed around the cell periphery. Cells treated with Phx for 12 h exhibited highly diminished expression of BCR at the cell surface. When cells were stimulated with anti-IgM antibody (M4), the surface IgM formed numerous membrane patches. Treatment with Phx reduced the patch formation of cell surface IgM after BCR stimulation.

Next, we examined the effect of Phx on IgM expression in intracellular sites. Cells were permeabilized with TritonX-100 and analysed by confocal microscopy. In control cells, many patches appeared with strong IgM immunostaining, whereas treatment with Phx reduced these patches containing IgM immunostaining. To distinguish whether the immunofluorescent patches were the vesicles which transport IgM from trans-Golgi network (TGN) to plasma membrane or the vesicles which degrade IgM, we performed double-staining with anti-TGN38 antibody, a marker for TGN. As shown in Figure 4i, the yellow staining indicated colocalization of TGN38 and IgM, suggesting that IgM immunofluorecent patches were located in the TGN.

Treatment with Phx reduces IgM secretion from B cells- To confirm whether the decrease in IgM in the TGN after treatment with Phx is due to the enhancement of IgM secretion into the medium, we analysed the amount of IgM in each culture medium using immunoblotting with anti-IgM. Treatment with Phx for 12 h diminished IgM secretion in a dose-dependent manner. These findings indicate that treatment with Phx reduces the secretion of IgM.

Treatment with a proteasomal inhibitor, MG-132, restores IgM levels-To clarify the mechanism of Phx-mediated down regulation of IgM, we tested the effects of a protease inhibitor, MG-132, on IgM, Syk and α-tubulin expression. IgM expression after Phx treatment was at about 45% of control levels. After treatment with MG-132, IgM levels became nearly equal to control, whereas Syk and α-tubulin levels remained unchanged throughout. In contrast with Phx, cycloheximide-induced down-regulation was not affected by MG-132. These results suggest that Phx specifically accelerates degradation of IgM.

DISCUSSION

The novel phenoxazine derivative, Phx has shown inhibitory effects on the proliferation of various cancer cell lines. In addition, it has been reported that Phx markedly reduces the growth of tumours in mice which have received a leukemia cell or a carcinoma cell transplant, without causing adverse effects such as loss of body weight and leukopenia. Thus, Phx is expected to be available as a new anti-tumour drug in the future. In this

paper, we further suggest that Phx has suppressive effects on immunoglobulin expression in B cells and might be useful as a new immunosuppressive agent.

We have demonstrated that treatment with Phx induces a reduction in IgM expression in B cells. The finding that IgM expression at the cell membrane was reduced by Phx treatment led us to expect that Phx either enhances the degradation of IgM or inhibits IgM production. IgM production is accomplished through RNA transcription and protein synthesis. When actinomycin D, an inhibitor of transcription, inhibited IgM production after 32 h of treatment, the other cellular proteins also decreased at the same time implying a general inhibition of protein synthesis. Although actinomycin D is known to inhibit the activity of DNA-dependent RNA polymerase to intercalate DNA, Phx has no DNA intercalating activity. This suggests that Phx has a mode of action different from actinomycin D. Cycloheximide, which inhibits peptideyl-translocation in ribosome, also reduced the level of proteins. Treatment with Phx decreased the expression of IgM, but not Syk or α-tubulin. However, B cells exposed to actinomycin D or cycloheximide exhibited a decrease in Syk, α-tubulin expression as well as in IgM expression. DT40 cells maintained in a serum containing medium continuously produce and secrete IgM. Treatment with Phx inhibited IgM production which led to diminished secretion of IgM.

It has been previously reported that Phx suppresses proliferation of cancer cells by inducing apoptosis. Also this study dose not exclude the possibility that Phx may promote apoptosis if DT40 cells are treated with Phx for a longer time. However the present results, that Phx specifically decreased IgM expression suggest that the mechanism of Phx-induced inhibition of proliferation is different from apoptosis. This is supported by the following observations: (1) Phx specifically decreased the amount of IgM, while it maintained the levels of other proteins; (2) In the presence of Phx (< 50mM) the percentage of nonviable cells remained at less than 2%, the same level as the untreated controls; (3) Apoptotic DNA degradation did not occur at 50mM Phx (12 h) whereas IgM expression decreased (data not shown). Since MG-132, which is a proteasomal inhibitor, increased IgM to the same level as the control (Figure 6), Phx has the effect of accelerating specific degradation of IgM. Phx may normalize the abnormal enhancement of immunoglobulin production in some diseases, such as autoimmune diseases and multiple myeloma. Phx is thus expected to be available for therapeutic purposes in the future.

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論文題目	A Novel Phenoxazine Derivative Suppresses Surface IgM Expression in DT40 B Cell Line (Phenoxazine 化合物 Phx は B 細胞において特異的な IgM 産生抑制効果を持つ)		
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（要旨は1，０００字～2，０００字程度）

B細胞では，B細胞抗原受容体（BCR)を介した刺激により，Lyn, Sykを始めとする細胞内チロシンキナーゼが活性化されるシグナルの伝達の結果，細胞増殖の制御及び異なる遺伝子発現の誘導が行われる．申請者はこれまで抗腫瘍活性が報告されてきた新種のPhenoxazine化合物Phx（2-amino-4, 4a-dihydro-4a, 7-dimethyl-3H-phenoxazine-3-one）が，その化学構造が免疫抑制作用を持つemodinと似ていることと，単核球のphytohemagglutinin刺激による増殖を抑えることから，免疫抑制剤としての可能性を予測し，B細胞を用いて研究を進めることを着想した．結果としてDT40 B細胞を用いて，IgM (BCR)産生能を特異的に抑制することを発見した．

申請者はニワトリDT40 B細胞にPhxを加えて培養し，抗IgM抗体を用いて，BCRの細胞内の量を調べた．Phx (100 mM)を加えて１２時間培養すると，BCRは顕著に減少した．同時にBCR下流のシグナルであるチロシンリン酸化も抑制されていることが抗リン酸化チロシン抗体を用いて分かった．IgMが減少する条件では他の蛋白質Syk, tublin, AKT, ERK, PLCg-2の量は変化しないことを確認した．共焦点顕微鏡により細胞表面と内部のBCRについて観察したところ，Phxによって顕著な減少が認められた．また，培養液中のIgMの分泌量の分析により，細胞内BCRの減少は分泌によるものではないことが示された．さらにその作用機序を明らかとするためにproteasome inhibitor MG-132を用いた結果，Phxの効果によって減少したIgM産生能が，回復することを見出した．以上のことから，申請者はPhxはIgMの分解を促進することによってIgM産生能を抑制していることを明らかにした．

申請者は優れた着眼点でこれまで抗腫瘍活性のみが知られていたPhx（2-amino-4, 4a-dihydro-4a, 7-dimethyl-3H-phenoxazine-3-one）がB細胞のIgM産生能を特異的に抑制し，B細胞抗原受容体（BCR）を介したシグナル伝達を抑えることを見出した．さらにPhxが免疫細胞受容体の分解を促進するという作用機構も明らかにした価値ある集積である。よって、本申請者は博士（医学）の学位を得るにふさわしい資格があると認める。