Assessment of GM-CSF receptors by real–time RT-PCR on cell lines expressing high and low affinity receptors and their relation to cytotoxic effect of chimeric protein (StxA1-GM-CSF)

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ABSTRACT

Immunotoxins, which are composed of both the cell targeting and the cell killing moieties are the new approach for targeted therapy of human disease .In all immunotoxins that GM-CSF has been used as cell targeting; only cell lines expressing high affinity receptor have been used for cytotoxicity studies. In the present study, various cell lines expressing high and low affinity receptors were used for assessment of the cytotoxic effect of hybrid chimeric protein. The expression of GM-CSF receptor (GM-CSFR) was quantified by real-time RT- PCR. The cell lines K562 and THP1 expressing high affinity receptor and MC-7, PC-3 and DU145 expressing low affinity receptor were used for this study. The chimeric hybrid protein was found to be toxic for various cell lines used in this investigation and cytotoxicity was more effective in cell lines bearing high affinity receptors. Overall, our results showed that the recombinant hybrid protein could have wide range of application on various cancer cell lines even cells bearing low affinity receptors for GM-CSF.

Keywords: StxA1-GM-CSF, GM-CSF receptor, Cytotoxicity, Real–time RT-PCR

INTRODUCTION

Immunotoxins, comprised of both the cell targeting and the cell killing moieties are the new approach for targeted therapy of human disease (1). Shiga-toxin produced by *Shigella* and *E.coli* is one of the members of ribosome-inactivating proteins (RIPs) and is composed of an enzymatic A subunit non-covalently associated with a B pentamer receptor-binding subunit. The toxicity of A-chain is due to its RNA-N-glycosidase activity, by which it brings about depurination of adenine at position 4324 in the 28S rRNA. This activity prevents formation of a critical stem-loop configuration, to which the elongation factor (EF) is known to bind during the translocation step of translation. The end result of this activity is complete inhibition of cellular translation (2). Granulocyte Macrophage-Colony Stimulating Factor Receptor (GM-CSFR) is composed of alpha and beta subunits. The isolated alpha subunit binds GM-CSF at low affinity (kd, 1 to 7 nmol/l). The isolated beta subunit dose not bind GM-CSF by itself, but in a complex with the alpha subunit forms a high-affinity receptor (kd, 20 to 100 pmol/l) (3, 4). In all immunotoxins, GM-CSF has been used as cell targeting and only cell lines expressing high affinity receptor have been used for cytotoxicity (5, 6) However no study dealing with the application of immunotoxin targeted toward cell lines bearing low affinity GMCSF receptor has been reported. We have already established a new immunotoxin, StxA1-GM-CSF, having a catalytic domain of Shiga-toxin, as a killing moiety and GM-CSF, as a cell targeting moiety (7). In the present study cell lines expressing high and low affinity receptors were used to compare the cytotoxic effects of the immunotoxin on these cells.

MATERIALS AND METHODS

Preparation of purified StxA1-GM-CSF protein

The recombinant protein expressed in *E. coli* (7), was purified by Pro Bond purification system kit (Invitrogen, USA) according to manufacture's protocol. The amount of protein was estimated by protein assay kit (BIO-RAD, USA).

Cell culture

HepG2 (human hepatoma), THP1 (Acute monocytic leukemia), K562 (Erythroid leukemia),

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DU145 (prostate carcinoma), PC-3 (prostate carcinoma) and MCF-7 (breast adenocarcinoma) were obtained from Cell Resource Center for Biomedical Research, IDAC, Tohoku university, Japan. These cell lines were grown in RPMI-1640 medium (Gibco-BRL, Germany) with 10% fetal bovine serum (Gibco-BRL, Germany).

RNA extraction

Total RNA from cell lines was extracted by Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality of RNA was determined by electrophoresis under denaturing conditions with 2 µg of RNA (8), (Fig 1).

cDNA synthesis

Reverse transcription was performed by SuperScript III reverse transcriptase (Invitrogen, USA) with 500 ng of total RNA followed by DNaseI (Invitrogen, USA) treatment and heat inactivation.

Real-time RT- PCR

Real-time RT-PCR analysis was performed by BIO-RAD icycler IQ, SA-THK Real-Time PCR system (BIO-RAD, Hercules, CA). Amplification was conducted using AB solute Syber green ROX mix (ABgene, surrey, UK) according to the manufacture's protocol. The PCR program included initial denaturation at 94 °C for 15 min followed by 40 amplification cycles consisting denaturation at 94 °C for 30 sec, annealing at suitable temperature for 30 sec and extension at 72 °C for 30 sec. Threshold cycle values were normalized by beta-actin expression. Primers used for the amplification of alpha subunit of GM-CSF receptor were as follows;
5'-CCCTTCTCTCTGACCAGCACC-3'and 5'-TTACTGAGCCTGGGTTCCACG-3', for Beta subunit were; 5'-GCGCTGCTACAACGACTAC-ACC-3'and reverse 5'-ACTCTGGCAGGGAA-TGACACATC-3' and for the normalization, beta-actin expression was examined and the primers were as follows;
5'-GCCAACCGCGAGAAGATGA-3' and 5'-CATCAGGATGCCAGTGGT-3'.

Cytotoxicity assays

The cytotoxic effect of StxA1-GM-CSF, on appropriate cell lines was determined by trypan blue dye exclusion and MTT assays (9). For trypan blue dye exclusion assay, 2.5×10^4 cells were seeded/well, in a 96-well plate. After 24 hrs, different concentration (10-160 ng) of StxA1-GM-CSF diluted in 200 µl of medium was added to each well. Following 24, 48, and 72 hrs, the number of viable cells was measured by trypan blue exclusion assay. For MTT assay, cells after 24, 48 or 72 hrs incubation with the StxA1-GM-CSF, were incubated with 10 µl of 0.5 mg/ml of 3- (4,5-Dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; sigma) at 37 ºC in 5% CO2 atmosphere for 4 hrs to allow MTT to be converted to formazon crystals by reacting with metabolically active cells. Reaction was stopped by addition of 10% SDS in 0.01M HCl and the cell viability was measured at 570 nm using a plate reader.

Statistical analysis

The results are expressed as mean ± SD of three independent experiments. Differences between groups were compared using ANOVA with Tukey-Kramer Multiple Comparison Test as a post test (* P<0.05, **P<0.01, *** P<0.001).

RESULTS

Real-time RT-PCR

The quality of extracted RNAs was checked by electrophoresis under denaturing condition. Two bands, 28S and 18S rRNA, were observed indicating the quality of extracted RNA (Figure 1).

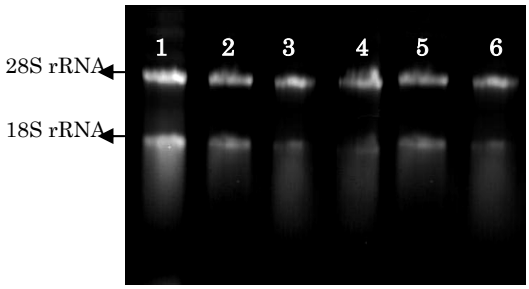

Figure 1. Electrophoresis of extracted RNA under denaturing condition. Two bands , 28S rRNA and 18S rRNA , indicating the quality of extracted RNA from different cell lines**,** i.e., HepG2 (lane-1), THP1(lane-2), K562(lane-3), MCF-7(lane-4), PC-3(lane-5), and DU-145(lane-6).

Out of 5 cell lines used; THP1 and K562 expressed both alpha and beta subunit of GM-CSF receptors. PC-3, MCF-7 and DU145 expressed only alpha subunit (Figure 2). HepG2 was used as negative control, lacking GM-CSF receptor. Among the cells bearing only alpha subunit, MCF-7 expressed more receptor than two other cells. K562 expressed more alpha subunit of GM-CSFR than THP1 but produced the same level of beta subunit. (Figure 2)

Cytotoxicity

To determine the sensitivity of the cells to StxA1-GM-CSF, cells were incubated with different concentration of hybrid protein for various times. K562 and THP1 cell lines treated with different amounts of A1-GM-CSF were found to be more

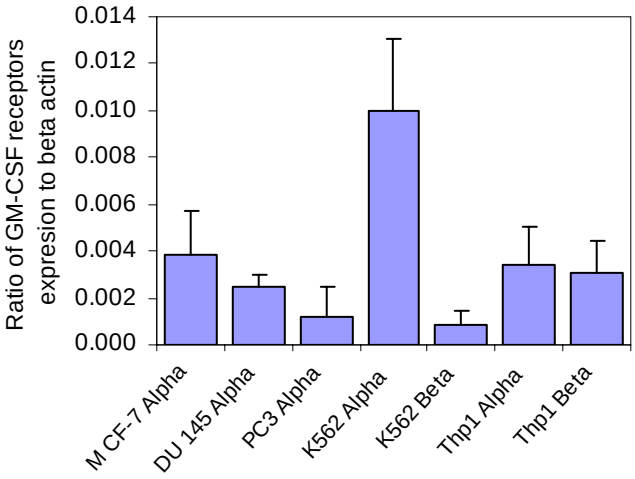

Figure 2. Expression of alpha and beta subunit of GM-CSFR by Real-time RT-PCR. THP1 and K562 expressed both alpha and beta subunit while PC-3, MCF-7 and DU145 expressed only alpha subunit. Bar is Mean±SD

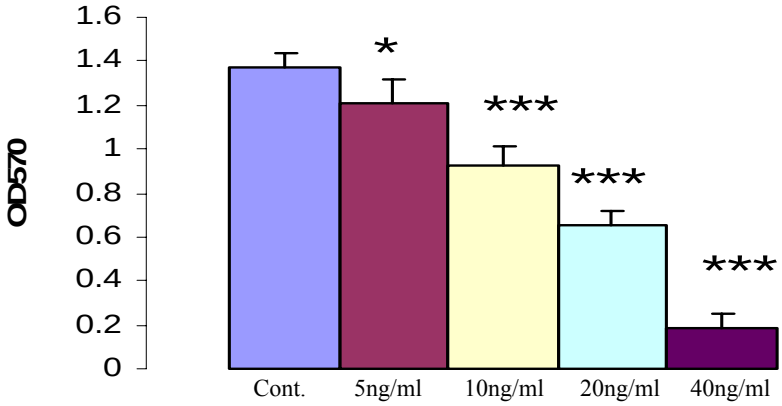

Figure 3. MTT assay, Effect of different concentration of StxA1-GM-CSF on K562 cell lines compared to Control (treated with PBS) for 24 hrs. Maximum cytotoxicity was observed at the concentration of 40ng/ml .(Mean±SD, *: P<0.05, ***: P<0.001)

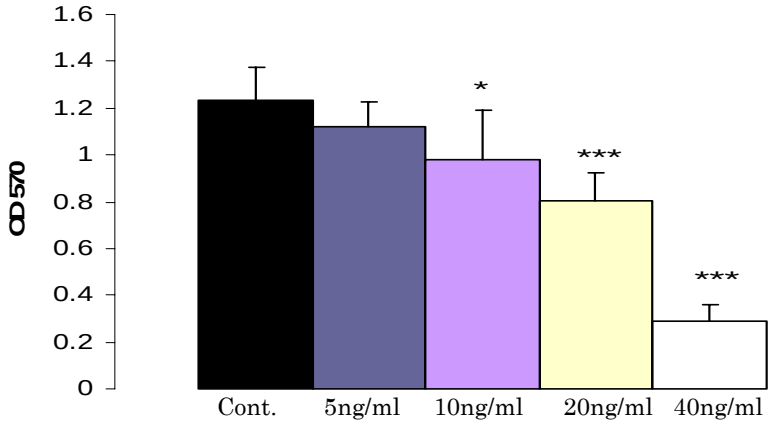

Figure 4. MTT assay, Effect of different concentration of StxA1-GM-CSF on THP1 cell line compared to Control (treated with PBS) for 24 hrs. Maximum cytotoxicity was observed at the concentration of 40ng/ml.(Mean±SD, *: P<0.05, **: P<0.01, ***: P<0.001)

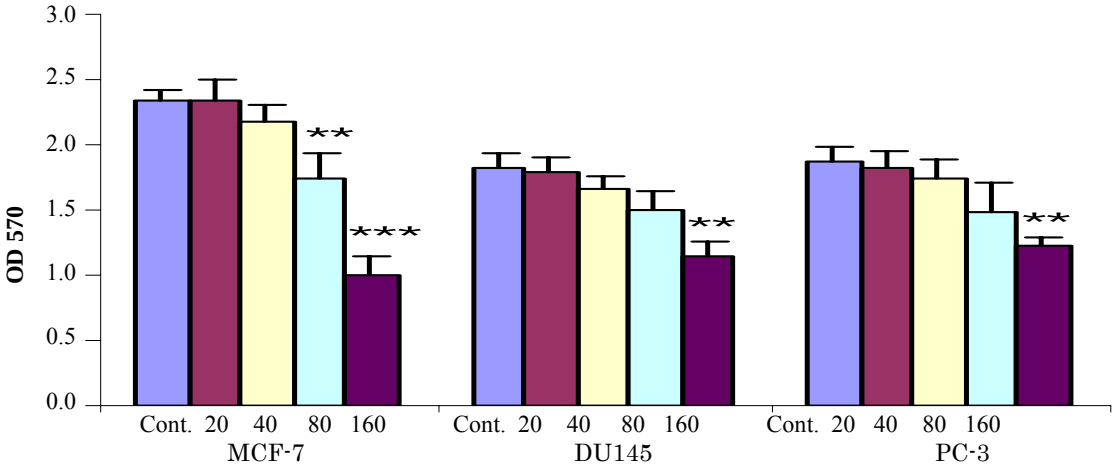

Figure 5. MTT assay, Effect of different concentration of StxA1-GM-CSF on cells bearing low affinity receptors for GM-CSF, MCF-7, DU-145 and PC-3 compared to Control (treated with PBS), for 72 hrs. MCF-7 was susceptible at concentration of 80 ng/ml and 160 but DU145 and PC-3 cell lines were susceptible only at concentration of 160 ng/ml of hybrid protein. (Mean±SD, **: P<0.01, ***: P<0.001)

susceptible to the fused toxin. The LC50s, of hybrid protein necessary for 50% inhibition of protein synthesis, were 18±3.5 ng/ml and 25.32 ± 4.26ng/ml for K562 and THP1 respectively. Maximum activity was observed at the concentration of 40ng/ml and the incubation time of 24 hrs (Figure 3 and 4). MCF-7 was susceptible at concentration of 80 ng/ml and 160 but DU145 and PC-3 cell lines were susceptible only at concentration of 160 ng/ml and incubation for 72 hrs (Figure 5). LC50s for MCF-7 cell line was 150 ng/ml±8 and for DU145 and PC-3 cell lines were higher than 160ng/ml. In both cytotoxicity assays, i.e., Trypan blue and MTT, HepG2 cell line was not susceptible to hybrid protein and was employed as negative control.

DISCUSSION

The real-time RT-PCR was used for detection and quantification of GM-CSF receptors on the cell lines used in the present study. To our knowledge this is the first report that alpha and beta subunit of GM-CSFR mRNAs were quantified by real-time RT-PCR. Using alpha subunit antibody for GM-CSF and RT-PCR it has been shown that PC-3, DU145, MCF-7, K562 cell lines express alpha subunits and THP1 expresses both alpha and beta (10, 11), which are high affinity receptors. Meanwhile we showed that K562 not only expresses alpha but also beta subunit. Sensitivity of real–time RT-PCR employed in the present study which was comparable to immuno-histochemistry and RT-PCR used by Chen *et al* (10), might be the probable reason for differences found in beta subunit expression in K562 cell line. Frankle *et al* (6), showed that DT-GM-CSF was ineffective on K562 in terms of cytotoxicity and apoptosis but StxA1-GM-CSF was found to be effective. Cells that express only low affinity receptor, i.e., PC-3, DU145 and MCF-7, showed less cytotoxicity and longer time compared to high affinity GM-CSFR bearing cells, i.e. HL60, U937, LS174T (12,13). In immunotoxins such as DT-GM-CSF and Pseudomonas-GM-CSF (5, 6), GM-CSF is used as a targeting moiety and have been tested for cell lines bearing high affinity GM-CSFR. The cytotoxic effect observed in this study by StxA1-GM-CSF on the cell lines expressing only low affinity receptor is novel, and has not been reported previously. Binding of GM-CSF to its cognate high affinity receptor causes rapid internalization (14). StxA1-GM-CSF most probably first enters through low affinity receptor during recycling of ligand-receptor and then exerts its cytotoxic effects. Receptors re-cyclization or down regulation both in the presence or absence of ligand activation has been reported (15). Different studies (5,6), have shown that different toxins fused to the same molecule as a targeting moiety showed different sensitivity on the same cell line. Similarly in our study, StxA1-GM-CSF showed more cytotoxicity on THP1 cell line compared to DT-GM-CSF (12). On the other hand density of receptor on cells, is shown to affect the sensitivity of the cell lines toward the immunotoxin (5, 6), i.e., when there are more receptors, more fused toxin enter the cells and there will be more cytotoxicity. DT-GM-CSF showed more cytotoxicity on U937 than HL60 cells, since the former is expressing more GM-CSFR. Low cytotoxicity of StxA1-GM-CSF on cells bearing low affinity receptor for GM-CSF also might be related to the high level secretion of GM-CSF by these cells (12). It has been shown

that co-incubation of DT-GM-CSF (5), with recombinant GM-CSF prevent cytotoxicity because of competition between recombinant GM-CSF and GM-CSF part of fused protein to its receptor. Overall our result showed the recombinant hybrid protein, StxA1-GM-CSF, is applicable for a wide variety of cancer cell lines even cells bearing low affinity receptors for GM-CSF. As it has been shown previously (12), this effect could be specific i.e. due to the presence of killing moiety of Shiga- toxin (A1), that exerts its effect through specific cell targeting domain i.e. GM-CSF present in cells.

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