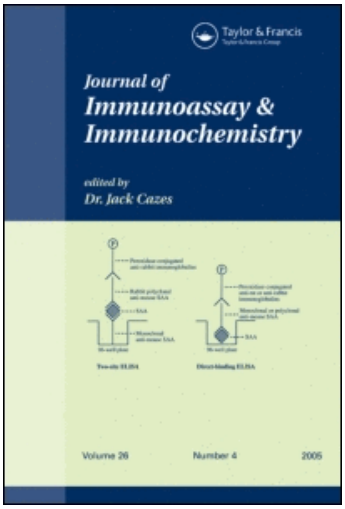

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□ *Interleukin-22 is a novel cytokine produced mainly in activated T cells. The elaborate biological functions of IL-22 in vivo are still widely unknown. In this report, we describe a rapid, simple, and reproducible in vitro cell-based bioassay for measuring the bioactivity of recombinant interleukin-22 (IL-22) to study the primary function of IL-22 in vivo. Human hepatocyte cell line (HepG2) was transfected with pSTAT3-Luc, a plasmid carrying the luciferase gene under the control of STAT3. After screening and selection, one stable clone was established which generates a strong response to recombinant human IL-22 (rhIL-22) stimulation in a dose-dependent manner. The cell showed ED50 of 17.0 ± 1.4 ng/mL (n = 15) to recombinant human IL-22. Pre-incubation of anti-IL-22 mAb with IL-22 recombinant proteins completely blocked the bioactivities. The assay can be completed within one day. The current assay provides a rapid analytical method to measure the biological activity of IL-22 in vitro.*

Keywords bioassay, HepG2, interleukin-22 (IL-22), luciferase assay, recombinant, STAT3

INTRODUCTION

Interleukin-22 (IL-22) was originally described as IL-10-related T cell-derived inducible factor (IL-TIF) with 22% amino acid homologous to IL-10.[1,2] IL-22 is expressed in activated T cells (CD4 + T helper 17), natural killer cells, and dendritic cells,[3] and upregulated in certain chronic inflammatory diseases.[4,5] IL (interleukin)-22 is an IL-10-related cytokine; its main biological activity known thus far is the induction of acute phase reactants in liver and pancreas.[6] IL-22 receptor expression is restricted in tissues, including intestinal epithelium, pancreas, liver, and skin, but absent in the hematopoietic and immune cells.[7] IL-22 receptor complex

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consists of IL22R1 and IL-10R2. The IL-10R2 is also the common chain for IL-10, IL-22, IL-26, and the IL-28/IL-29 receptor complex.[8] IL-22 binds to its membrane receptor, resulting in the rapid activation of JAK1 and Tyk2, which then tyrosine-phosphorylates STAT1, STAT3, and STAT5.[9] Recent studies have suggested that IL-22 exhibits both pro- and anti-inflammation activities.[10,11]

To accomplish further study on biological roles of IL-22 *in vivo*, we have expressed IL-22 recombinant proteins from human, mice, and several other species. It is crucial to have a rapid and reliable bioassay for monitoring the expression and separation of recombinant IL-22 proteins. Therefore, we developed a reporter-based stable cell line to measure IL-22 bioactivity. This cell line, HepG2-C16, is sensitive, rapid, and reproducible to detect bioactivities at ng/mL levels of recombinant IL-22.

EXPERIMENTAL

Reagents

pSTAT3-TA-Luc plasmid was purchased from Takara Bio Inc. (Shiga, Japan), Dulbecco's Modified Eagle's Medium (DMEM), FBS, Lipofectamine 2000 and opti-MEM I serum free medium were purchased from Invitrogen Life Technologies (Gaithersburg, MD, USA). Luciferase 1000 Assay System was from Promega Corporation (Madison, WI, USA). Recombinant human IL-1β, IL-2, IL-3, IL-6, IL-7, IL-11, TNF-α, IFN-γ, GM-CSF, MCP-1 were from R&D systems Inc. (Minneapolis, MN, USA). Recombinant human IL-22 (rhIL-22) and recombinant mouse IL-22 (rmIL-22) was produced in *Escherichia coli* as described previously.[1]

Cell Culture and Transfection

HepG2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% FBS (fetal bovine serum). HepG2 cells were co-transfected with pSTAT3-TA-Luc and pcDNA3.1 as suggested by the kit manufacturer (Invitrogen). Twenty-four hours after transfection, cells were screened with G418 at the concentration of 400 μg/mL for 4 weeks. After cloning of the G418 resistant cells, a few dozen of the clones were screened for luciferase activity.

IL-22 Bioassay

Tranfected HepG2 cells were plated at 4×10^5 cells/mL in 96-well plate, 100 μL per well. Twenty-four hours later, cells were treated with

recombinant cytokines at a different concentration. Negative control wells were added with 10 μL of growth medium. After 4 hours of incubation, the medium was removed and luciferase assays were performed using Luciferase 1000 Assay System (Promega). Activity was determined using a PerkinElmer Victor III reader. Luciferase activity was reported as relative luciferase units (RLU).

Data Analysis

The median effective dose (ED50) was calculated using non-linear regression analysis (SigmaPlot v10.0, Systat Software Inc. San Jose, CA, USA). The levels of significance ($^{**}P < 0.01$, $^{*}P < 0.05$) were calculated by Student's *t*-test.

RESULTS

Assay Development

HepG2 cells were co-transfected with pSTAT3-TA-Luc plasmid containing a luciferase reporter gene and pcDNA3.1 plasmid containing the neomycin resistance gene. A dozen clones were established after screening by G418 for 4 weeks. The clone 16 was found to be the most sensitive upon stimulation with recombinant human IL-22 (Fig. 1a) (7-fold increase compared to the base level signal) and was named as HepG2-C16. HepG2-C16 showed approximately 18-fold sensitivity compared to the tranfected pool. A linear increase of luciferase activity to IL-22 was observed between 3 ng/mL and 300 ng/mL ($r^2 = 0.99$). In 15-repeated independent experiments; the mean ED50 was 17.0 ± 1.4 ng/mL.

Validation of the Assay

It has been reported that Colo205 cells could release IL-10 in response to IL-22 stimulation.[12] Therefore, we chose the Colo205 cell system to validate the current IL-22 bioassay (Fig. 1b). Recombinant IL-22 preparations were incubated with Colo205 cells for 24 hours. The supernatant was assayed for IL-10 by using an ELISA kit. Results showed that Colo205 cells produced IL-10 in response to IL-22 at dose-dependent manner. The Colo205 cells, compared to HepG2 cells, were more sensitive to IL-22 with ED50 of 0.24 ± 0.08 ng/mL ($n = 3$). But, the response of HepG2-C16 cells to IL-22 was more rapid and robust, and meets the need for protein processing development.

Then we determined the selectivity of HepG2-C16 against different cytokines including recombinant human IL-1β, IL-2, IL-3, IL-6, IL-7,

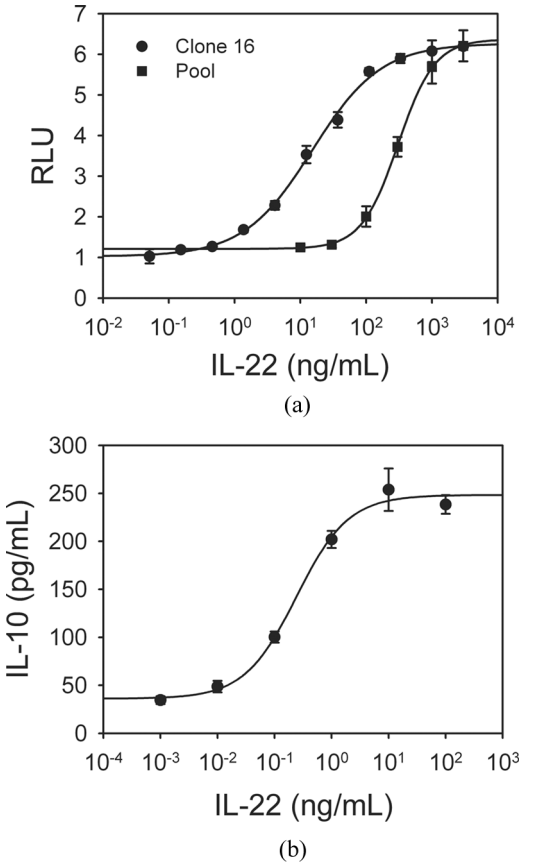


FIGURE 1 Response of different cells to rhIL-22. (a) Response of transfected HepG2 cells to rhIL-22. (b) Response of Colo205 cells to IL-22. Colo205 cells were treated overnight with different concentration of rhIL-22. The amount of IL-10 in supernatants was measured using an IL-10-specific sandwich ELISA assay.

IL-11, TNF-α, IFN-γ, GM-CSF, MCP-1, since some of these cytokines are capable of activating STAT3 pathways.[13,14] Results showed that HepG2-C16 generated the strongest response to IL-6 (approximately 30-fold over negative control). In addition, IL-1β also showed significant response in this assay. The rest of the cytokines did not result in significant response (Fig. 2). In the routine bioassay of IL-22, rhIL-6 was used as a positive control.

To further validate the assay using HepG2-C16, neutralizing polyclonal antibody to rhIL-22 was added to the testing wells. Results showed that adding anti-IL-22 antibodies blocked the induction of luciferase activity in a dose-dependent manner (Fig. 3). Furthermore, adding the same concentration of anti-IL-22 antibodies to cultures containing IL-6 had no effect on IL-6-induced luciferase activities. These results demonstrated that

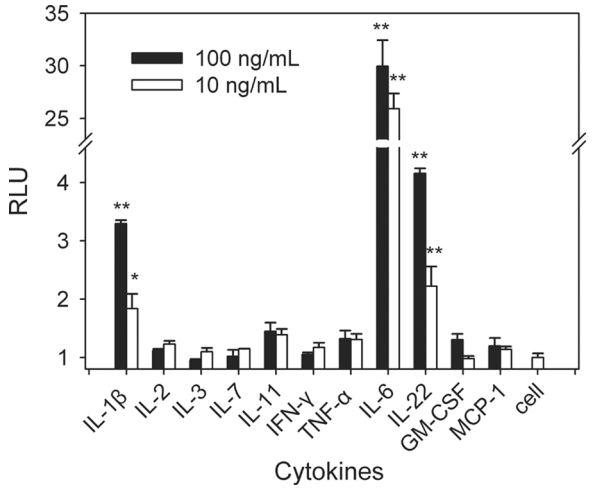


FIGURE 2 Effect of different cytokines on STAT3 activation in HepG2-C16 cells. Cells were stimulated with different cytokines at the final concentration of 100 ng/mL (■) and 10 ng/mL (□). Cell: untreated control.

HepG2-C16 was specifically responsive to IL-22 and could be blocked with anti-IL-22 antibodies.

Response of HepG2-C16 to Different Forms of IL-22

We prepared recombinant IL-22 from mouse and human, with and without pegylation. The rhIL-22, rmIL-22, PEGylated rhIL-22, PEGylated rmIL-22, and rhIL-22 dimer bioactivities were evaluated (Fig. 4). All the recombinant IL-22 proteins were able to induce the luciferase activities, however, at different potencies (ED50 of 17.0 ng/mL, 277 ng/mL,

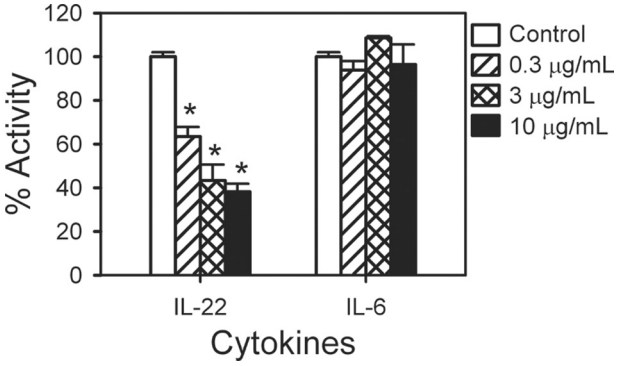
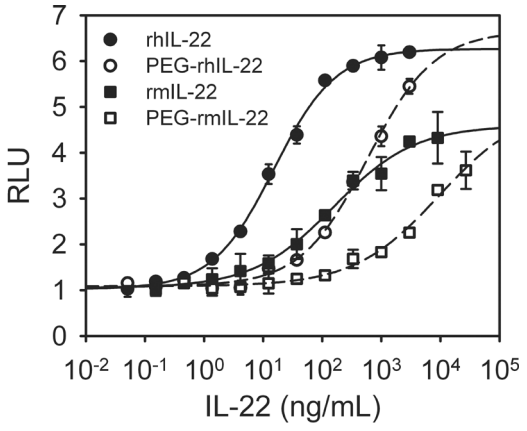
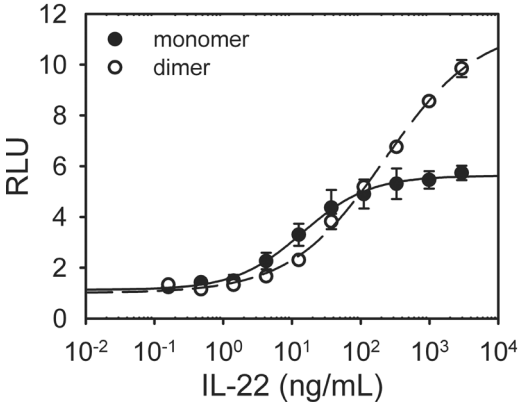


FIGURE 3 IL-22 neutralizing antibody inhibits IL-22 activated luciferase activity. Anti-IL-22 neutralizing antibody was tested at 0.3 μg/mL, 3 μg/mL and 10 μg/mL in cultures containing 10 ng/mL of rhIL-22 or rhIL-6, respectively.



(a)



(b)

FIGURE 4 Response of HepG2-C16 to different recombinant IL-22 proteins. (a) rhIL-22 (●), PEG-rhIL-22 (○), rmIL-22 (■) and PEG-rmIL-22(□). (b) IL-22 monomer (●) versus IL-22 dimer (○).

157 ng/mL, 9.5 μg/mL, and 225.0 ng/mL, respectively). The assay was more sensitive to rhIL-22 than to rmIL-22. Pegylation of IL-22 resulted in a significantly decreased response in the assay. Dimerization of rhIL-22 did not show plateau response in the current experimental conditions, suggesting a different mechanism of activating STAT3 that may occur at the interaction of IL-22 receptors with its ligand (Fig. 4b).

DISCUSSION

Based on the evidences of IL-22 activating STAT1, STAT3, and STAT5 in HepG2 cells,[15] we successfully developed a specific, rapid and reproducible bio-assay for studying the biological activities of recombinant IL-22

preparations. Nagalakshmi described a quantitative IL-22 bioassay based on the fact that IL-22 induced IL-10 secretion in Colo205 cells.[12] However, the assay is time-consuming and involves multiple steps and the introduction of multiple variables.

We also found differences in activity between different recombinant IL-22 proteins. Both human and mouse IL-22 have 179 residues and share 79% amino acid sequence identity.[16] Human and mouse IL-22R1, IL-10R2 share 71.9% and 64.2% identity in amino acid sequence, respectively. These discrepancies suggest that rmIL-22 could activate STAT3 in human derived HepG2 cells, but is less active than rhIL-22. For this reason, murine derived hepatoma cells H4IIE maybe more sensitive for rmIL-22 bioassay.[9]

Conjugation of proteins to polyethylene glycol is a widely used strategy to improve therapeutic potential of recombinant proteins. Pegylation can influence the binding affinity of proteins to cellular receptors and cause a decrease in their *in vitro* activity.[17] Here, we have found that pegylation of IL-22 resulted in a significant decreased response. However, this negative effect can be offset *in vivo* by a prolonged half-life and reduced immunogenicity (data not shown).

IL-22 can form homodimer through hydrophobic interaction.[16] Interestingly, the dose-response curve of IL-22 dimer and monomer do not parallel (Fig. 4B), which suggests that they might activate STAT3 in a different way. De Oliveira Neto reported that IL-22 could form a V-shaped dimer that could be recognized by two IL-22R1 chains.[18] Here, we have demonstrated that IL-22 dimer could activate STAT3 in a different manner that remains to be further explored.

CONCLUSION

In summary, the current described cell-based IL-22 bioassay is helpful in testing preparations of purified recombinant IL-22. This method could become a valuable tool in evaluating biological potency of different recombinant IL-22 proteins, and optimizing the protein processing conditions. Moreover, the bioassay can be used to identify novel modulators in IL-22 signaling transduction pathways.

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