Abstract

IL-10 is a pleiotropic growth and differentiation factor of B cells. To explore its gene regulation in the presence of other lymphoid cells, we constructed the enhanced green fluorescent protein (EGFP)-based reporter plasmids under the control of IL-10 promoter sequences spanning from -1212 to -19 region. The expression of EGFP was observed directly by fluorescent microscopy and quantitatively measured by flow cytometric analysis and fluorescence spectrophotometer. By transient expression experiments, IL-10 promoter activities were high in BJAB, and U937; low in Jurkat; while not detectable in MC116, Molt-4 and HL-60. Stable cells expressing EGFP driven by CMV and IL-10 promoters have been established in Jurkat and BJAB cells. Immunomodulating agents including mitogens, dexamethasone, cyclosporine A, and staurosporine, or by activated Ha-Ras mutant gene, or with different cells, blood, and tissue mixtures, were used to observe IL-10 promoter activities. The results in multiple experimental conditions showed that Jurkat and BJAB cells had different IL-10 promoter activities. In Jurkat and BJAB cells, the expression of IL-10 promoter was mainly due to forskolin treatment and coculture with human peripheral blood mononuclear cells. Collectively, stable cells mounted with EGFP-based reporter plasmids might provide a convenient screen system for agents affecting the CMV and IL-10 promoter activities. These data should further the understanding of how cells interact with each other.

一、中文摘要

介白素十號(interleukin-10(IL-10))是體內一種重要的細胞激素，它在免疫反應中扮演抑制發炎反應及抑制免疫能力的角色。主要由體內的T淋巴球細胞、B淋巴球細胞與巨噬細胞所製造分泌。它能抑制T細胞、NK細胞與巨噬細胞的活性；也可以增強B細胞與mast cells的增生與分化。本計劃中我們選殖了含有不同IL-10 promoter片段，並將它們接入載體：-19到-1212(pEGFP10p)、-19到-746(pEGFPd3)與-19到-558(pEGFPd)，使IL-10 promoter片段下游接著綠色熒光蛋白(enhanced green fluorescent protein, EGFP)報告基因。我們建立起帶有質體並穩定表現EGFP的Jurkat與BJAB穩定細胞株，並以mitogen，forskolin，dexamethasone，cyclosporine A，和staurosporine，或以activated Ha-Ras mutant gene，或與不同的細胞、血液混合培養，來觀察IL-10 promoter的調控。結果在多種藥物處理下Jurkat與BJAB的IL-10 promoter activities受到調控的情形是不同的。在混合培養實驗中，活化的Jurkat、BJAB，與U-937細胞可以增強BJAB穩定細胞株的IL-10 promoter的活性，但在Jurkat穩定細胞株中則無顯著影響。而SLE病人與健康人的PBMC和plasma對於IL-10 promoter活性也有不同的影響。因此，穩定細胞株可以反應出Jurkat與BJAB的IL-10 promoter有不同的調控機制，是一個方便有效的偵測系統。

關鍵詞：介白素十號、啟動子、報告基因
Defining the dynamic regulation of a gene in living cells is required for further application of the knowledge for the interest of human being. Several reporter systems have been developed to study the promoter activity of the test DNA region in appropriate cells. Among those reporter proteins, green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is stable, emits green light upon exposure to UV and can be monitored noninvasively in living cells 

These properties constitute a GFP-based reporter system being well suited for monitoring gene expression in real time. Moreover, the GFP reporter gene can serve to assay the communication between cells, in which target and effect cells can be distinguished easily by fluorescence regardless how similar they are in shape.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine and B-cell proliferation factor and has been implicated in autoimmunity, tumorigenesis and transplantation tolerance. A number of virus and viral gene products can initiate IL-10 expression. Previous study using luciferase-expression assay has identified some regulatory sequences for the constitutive or EBV-infection associated expression of IL-10 in human B cell lymphoma. While chemical agents were usually used to study promoter activities in vitro, the microenvironment in situ and the interaction of immune cells with neighboring cells might determine most immune reactions in vivo. Improper cross-regulation by local cytokines secreted from Th1 and Th2 cell subsets may be detrimental to the host during the course of infection and this may not be reflected by dysfunction of individual cells.

In this study we attempted to explore the regulation of IL-10 promoter in the presence of different types of lymphoid cells. Take the advantage of EGFP protein mentioned above, we constructed reporter plasmids, in that the expression of enhanced green fluorescent protein (EGFP) is under the control of IL-10 promoter sequences. Stable cells expressing EGFP driven by IL-10 or CMV promoter were established in Jurkat and BJAB cells representing T and B cells, respectively. The promoter activities were analyzed in those stable cells after cultured together with different cell lines including T, B, and monocytes. Results obtained will assist in the understanding of dynamic expression of IL-10 and relationship with other cells of the immune system generally.

Expression of EGFP in cells.

After transfected with pEGFP-N1 plasmid, all cell lines tested expressed high level of EGFP. The number of EGFP-positive cells visualized under fluorescence microscope or FACSscan reached maximal around 36-48 h post-transfection, then decreased gradually in growth condition without G418 selection pressure. Some sorts of cells used in this study were able to support the expression of EGFP driven by IL-10 promoter. The IL-10 promoter activities were high in BJAB, and U937 as reflected by strong EGFP expression; low in Jurkat; but not detectable in MC116, Molt-4 and HL-60. The EGFP-expressing cells derived from Jurkat and BJAB cells emitted green light under fluorescent microscopy and appeared morphologically similar to parental cells. Difference in fluorescent intensity of
cells carrying different promoters could be easily seen under fluorescent microscope. Analyses with fluorescence spectrophotometer and FACScan have been compared for the quantification of EGFP in BJAB cells. They revealed same results using materials of either transient expression or stable cells. Although EGFP measurement by fluorescence spectrophotometer showed low background value, it required relative large amount of cell materials for a reliable detection.

Stable cells expressing EGFP driven by CMV or IL-10 promoters have been established in Jurkat and BJAB cells. After selection for 3 months with G418, only about 10-20% of G418-resistant cells expressed EGFP. The levels of EGFP in stable cells were slight lower than those deduced by transient expression experiment. About 26% of BJAB cells expressed EGFP with a mean intensity of 1513 (FL1: 400-1023) in transient expression 36 h-posttransfection with pEGFP-N1. In bulk culture of pEGFP-N1-stable cells after G418 selection, about 10% cells expressed EGFP with a mean intensity of 1414 (FL1: 400-1023). Similar results were observed for pEGFP10p. About 23% of BJAB cells expressed EGFP with a mean intensity of 241 (FL1: 400-1023) in transient expression 36 h-posttransfection. In bulk culture of pEGFP10p-stable cells after G418 selection, about 13% cells expressed EGFP with a mean intensity of 119 (FL1 400-1023). We failed to establish stable cells with U937 cells, since those EGFP-positive cells died off in about a week posttransfection.

**IL-10 promoter activities in activated T cells.**

The IL-10 promoter activities in activated Jurkat and Molt-4 cells had been explored by transient expression experiments. Cells were treated with PMA or A23187 or PMA plus A23187. None of those treatments altered the levels of EGFP in Jurkat cells transfected with pEGFP10p, that about 335±10 relative fluorescence was detected by FACScan analysis. In addition, those treatments were not capable to induce the expression of EGFP in Molt-4 cells transfected with pEGFP10p, pEGFPd3 and PEGFPd. They had only background fluorescent emission similar to that detected in control cells carrying pEGFP.

Expression of EGFP in stable cells treated with dexamethasone, forskolin, cyclosporin A, staurosporine and activated Ha-Ras mutant.

The expressions of EGFP in treated cells were compared and expressed as relative mean fluorescence value (Fig. 1). Neither of CMV promoter- nor IL-10 promoter-driven EGFP in Jurkat cells was affected by dexamethasone. However, dexamethasone decreased the expressions of EGFP driven by IL-10 promoter in BJAB cells. The IL-10 promoter activities of pEGFP10p, pEGFPd3 and pEGFPd were inhibited to a similar degree by dexamethasone. In contrast to dexamethasone, forskolin enhanced the CMV and IL-10 promoter activities in some cell lines. The expressions of EGFP in BJAB cells, regardless of the promoter origin, were slightly increased. The Jurkat cells carrying pEGFP-N1 and pEGFP10p expressed elevated levels of EGFP upon forskolin treatment (p<0.01). While, the stimulatory effect of forskolin was not observed in Jurkat cells carrying pEGFPd3, pEGFPd or pEGFP plasmids. Treatments with cyclosporin A and staurosporine did not significantly influence the expressions of EGFP in those stable cells.

The expressions of EGFP in various stable cells after transfection with activated Ha-Ras mutant gene were also compared.
Transfection of activated Ras mutant gene into Jurkat stable cells enhanced significantly only the expression of EGFP driven by CMV promoter but not those by IL-10 promoter. The expressions of EGFP in BJAB stable cells were not affected by activated Ha-Ras mutant gene, neither CMV promoter-driven nor IL-10 promoter-driven.

The effects of various agents on the expression of IL-10 were also determined directly in Jurkat and BJAB cells by RT-PCR. Dexamethasone at $10^{-6}$M suppressed the transcripts of IL-10 gene in Jurkat and BJAB cells. Jurkat cells showed enhanced amount of IL-10 transcripts upon forskolin treatment, while BJAB cells did not.

Expression of EGFP in stable cells cultured with PBMC, T, B or monocytic cells.

To explore the dynamic communication between different lymphoid cells, EGFP-stable cells were cultured with PBMC, T, B or monocytic cells (Fig. 2). Cells were then harvested at 48 h for the determination of EGFP. The CMV promoter activities in most BJAB cells and Jurkat cells were not altered significantly by treatments with activating agents or coculture with lymphoid cells of different origin. Exceptions were coculture with PBMC and Jurkat cells, activated or non-activated, that they were able to suppress the CMV promoter activity in BJAB cells. LPS could elevate slightly the levels of IL-10 promoter-driven EGFP in BJAB cells. Stable Jurkat cells carrying plasmids, in that EGFP gene was driven by various regions of IL-10 promoter, did not change the EGFP levels when mixed with BJAB or U-937 cells as compared with controls. Interestingly, the expression of EGFP in Jurkat stable cells carrying pEGFP10p was enhanced significantly by PBMC isolated from five healthy individuals ($p<0.01$). While, PBMC did not affect the expressions of EGFP in Jurkat stable cells carrying pEGFP-N1, pEGFPd3 or pEGFPd.

四、討論

Using the reporter system we present here, differential regulations of IL-10 and CMV promoter activities were observed in T and B cells. CMV promoter is well known for its good ability in driving gene expression in most mammalian cells. As expected, pEGFP-N1-carrying cells (detecting CMV promoter) showed higher fluorescence intensity than that of pEGFP10p-carrying cells (detecting IL-10 promoter). Although IL-10 can be expressed in a variety of tissues, only three of six cell lines tested in this study were able to support the expression of EGFP under the control of IL-10 promoter. This observation was further confirmed by RT-PCR analysis that few IL-10 transcripts were detected in those EGFP-negative cell lines. It is to note that after selection by G418, about 10-20% of those G418-resistant stable cells, regardless either from Jurkat or BJAB, emitted green light. This differed from the stable cells established from glioma cells, that more than 80% stable cells carrying pEGFP-N1 plasmid expressed high level of EGFP (unpublished data of BCY). We failed to increase the number of EGFP-positive cells by elevation of G418 dose. What factor leads only a portion of stable cells to express EGFP awaits further study. In spite of many EGFP-negative cells existed in the bulk culture of stable cell lines, the expressions of EGFP in those EGFP-positive cells still reflected a well regulated pattern of promoter activities. The levels of EGFP detected by FACScan were positively correlated with the amount of IL-10 transcripts, that both levels
were high in BJAB and U-937 cells but low in Jurkat cells. Moreover, the patterns of EGFP expression in those stable cells we established were comparable with the transient expression cells in terms of mean fluorescence. Although the mean fluorescence of stable cells was slightly weaker than those of transient expression cells received same plasmids, the level of EGFP driven by CMV promoter was always higher than those by IL-10 promoters. This indicates that the expressions of EGFP reflected closely the activities of IL-10 promoter in those stable cells and that G418 selection did not alter the promoter activities.

Characterization of the promoter and 5' flanking regions of IL-10 gene has demonstrated the presence of positive and negative regulatory segments. In this study, the EGFP-reporter plasmids carrying different DNA fragments 5'-upstream of IL-10 gene provided a convenient way to show the dynamic activities of IL-10 promoter. The constitutive expressions of EGFP were comparable in Jurkat and BJAB cells received reporter plasmids and this expression pattern is in agreement with the study using luciferase as a reporter deduced from B cell lines. Yet, different treatments might have distinct effect on the EGFP expressions when particular IL-10 promoter fragments were used. For instances, forskolin and coculture with PBMC from health persons activated only the expression of EGFP in Jurkat cells carrying pEGFP10p plasmid but not others indicating the existence of positive regulatory sequences in this region. Forskolin is a cAMP agonist and may activate protein kinase A, which induces the expression of IL-10. Our results confirmed the involvement of cAMP pathway in IL-10 production of T cells at transcriptional level. The region -1219bp to -796bp contains some putative regulatory elements including Ets transcription factor binding site, estrogen receptor element, NF-IL-6 site and ISRE-like element. To our surprise, cAMP responsive element in IL-10 promoter is located at -409bp, but not in the region being responsive to forskolin in Jurkat cells. Particularly noteworthy is this region mediated the modulatory effect of PBMC on Jurkat cells (this study) and polymorphism in this region may be associated with abnormal IL-10 expression. Which binding site is responsible and whether multiple factors are needed for the expression of IL-10 in Jurkat cells require further investigation. As mentioned earlier, IL-10 can shape the immune status. Understanding the regulation of this region might give insight to the mechanism for the IL-10-associated disorders.

The effects of agents on IL-10 promoter we observed have been further confirmed by detecting directly the IL-10 transcripts in Jurkat cells. Analysis on IL-10 transcripts came to the same conclusion on the effects of most treatments except treating Jurkat cells with dexamethasone. The expression of EGFP driven by IL-10 promoter fragments up to -1219bp did not response to dexamethasone in Jurkat cells. However, dexamethasone at 10µM reduced significantly the amount IL-10 transcripts in Jurkat cells. Besides, dexamethasone is reported to inhibit the IL-10 of human PBMC. The discrepancy between EGFP and IL-10 transcript levels in Jurkat cells after dexamethasone treatment suggests that the dexamethasone-responsive element on IL-10 promoter in Jurkat cells may locate 5'-upstream -1219bp. This speculation is supported by the finding that some regulatory segments are located 3-4 kilobases.
upstream of the transcription initiation site of IL-10. Besides, our results indicated also that different dexamethasone-regulatory factors are used in B and T cells to regulate the IL-10 promoter.

IL-10 production by human T cells is thought to be a secondary event in cell activation and may serve as a feedback mechanism within the immune system to limit ongoing immune activation. In the current study we noted that PBMC from healthy persons acted positively on the IL-10 promoter activity in Jurkat, but not BJAB cells. This implies that modulation of IL-10 in T cells upon cross-talk with PBMC may contribute to maintenance of immune balance. Although viral IL-10 is critical to enhance the human IL-10 production, coculture with two IL-10 producing cells, BJAB and U937, did not further activate the IL-10 promoter activity in Jurkat cells. Thus, IL-10 is not necessary always to induce IL-10 production in an autocrine manner for some cells. Our finding partly elucidate the importance of cell to cell communication and the consequent microenvironment within a cytokine network. Similarly, various treatments may exert distinct effects on the activity of CMV promoter in particular cells. Overexpression of Ras activity enhanced the CMV promoter activity in Jurkat cells, but not BJAB cells. The CMV promoter activities in BJAB cells could be inhibited to various levels by coculture with Jurkat, or PBMC. Collectively, this EGFP-based monitoring system for living cells revealed a complicate regulation of IL-10 and CMV promoters and might provide a useful tool to elucidate the dynamic regulation of gene in living cells.

五、参考文献
9. Benjamin D., Knobloch T.J. &


15. BROSKI A.P. & HALLORAN P.F. (1994) IL-10 mRNA is detectable by PCR in many organs of normal mice and is largely T and B cell-independent. Tissue distribution of IL-10 mRNA in normal mice. Evidence that a component of IL-10 expression is T and B cell-independent and increased by irradiation. *Transplantation* 57, 582.


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Fig. 2

Expression of EGFP in stable cells cultured with PBMC, T, B or monocytic cell lines.

A. Jurkat derived stable cells

B. BJAB derived stable cells