247 marrow, were first identified as bone-forming progenitor cells from rat marrow.[72] MSCs represent a very small fraction, 0.001−0.01% of the total population of nucleated cells in marrow.[73] They have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage, and muscle. Recently, it has been reported that MSCs can differentiate into other lineages, such as neurons,[74] hepatocytes,[75] and insulin-producing cells.[76] Therefore, MSCs have attracted a great deal of interest because of their potential use in regenerative medicine and tissue engineering. To date, MSCs could be differentiated in vitro into proper lineages via a change in the culture conditions.[77] Another method for the in vitro differentiation is to genetically modify MSCs.[78,79] Although exogenous gene transfer into human MSCs (hMSCs) has been reported by using a conventional Ad vector, its transduction efficiency is quite low due to the scarcity of CAR.[80,81] Therefore, hMSCs have been transduced with high titers (more than 1000 infectious units/cell) of Ad vectors.[80,81] Fiber-modified Ad vectors have been applied for hMSCs to improve the transduction efficiency.[79,82,83] hMSCs infected with the AdRGD vector containing the BMP2 gene produced larger amounts of BMP2 than cells infected with the conventional Ad vector and efficiently differentiated into the osteogenic lineage.[82,83] Highly efficient transduction of hMSCs was achieved with tropism-modified Ad5 vectors carrying fiber shaft domains and knobs of different serotypes of Ad, such as Ad16, Ad35, or Ad50.[84] In a systematic comparison with various types of fiber-modified Ad vectors, the AdK7 vector is the most efficient for hMSCs and exhibited a 460-fold higher transduction efficiency than the conventional Ad vector.[79] The AdRGD vector or the Ad vector containing the Ad35 fiber (AdF35) exhibits a 16 or 130 times higher transduction efficiency, respectively, than the conventional Ad vector.[79] hMSCs are found to express CD46, which is the primary receptor for Ad35, but not CAR.[79] In conclusion, the AdK7 or AdF35 vector is the most appropriate for the transduction of hMSCs (Figure 3B). 283

Gene Transfer into Hematopoietic Stem Cells. Hematopoietic stem cells (HSCs) are capable of self-renewal and multilineage differentiation into all mature blood cells.[85] HSCs comprise only 0.01% of the whole bone marrow, the tissue in which they primarily reside.[86] Efficient transduction into HSCs would afford the opportunity to treat a number of hematopoietic disorders and would be a powerful tool for 290

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the study of the proliferation, differentiation, and trafficking of HSCs. Although the retroviral and lentiviral transduction of HSCs to achieve stable gene expression has been established,[87,88] stable expression is not always desirable. For example, stable expression of MDR1 gene results in HSC expansion but can cause leukemia upon transplantation to recipient mice.[89] As the Ad vector mediates the exogenous gene expression transiently, this vehicle can be safe for gene therapy. However, the application of conventional Ad vectors for the transduction into human CD34+ cells, which contain a population of HSCs, has been limited because CAR is not expressed at sufficient levels in human CD34+ cells.[90,91] It has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34+ cells and hematopoietic cell lines.[90,92] We showed that the Ad35 vector, which is composed from the whole Ad35, achieved higher levels of transduction efficiency in human bone marrow CD34+ cells than both conventional Ad5 vectors and AdF35 vectors.[39,93] The expression level of reporter genes in the CD34+ cells transduced with the Ad35 vector was 12−76 and 1.4−3 times higher than that in the cells transduced with the Ad5 and AdF35 vectors, respectively.[39] The transduction efficiency of the Ad35 vector was slightly higher than that of the AdF35 vector, although the reason remains unknown. CD46 is ubiquitously expressed in almost all human cells, including human cord blood CD34+ cells.[94] Therefore, human CD34+ cells would be considered to be a suitable target for the Ad35 vector (Figure 3C). As a result of the systematic comparison of promoters with Ad35 vectors, significantly higher transduction efficiencies were achieved with the EF-1α, CA, and CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi) promoters. In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34+ cells and the immature subsets.[93] In mice, a population of mouse bone marrow highly enriched for HSC, called side population (SP) cells, has been reported to be transduced with the conventional Ad5 vector.[95] This suggests that pure mouse HSCs might express CAR on the cell surface. Further studies are needed to clarify this. The Ad vector-mediated transduction of hematopoietic regulator genes, such as HoxB4,[68,69] Bmi-1,[96] or SCL/Tal-1,[97] into HSCs may be effective for therapeutic use such as HSC expansion, although the Ad vector expressing HoxB4 was unsuccessful because of unexpected HSC differentiation due to its high transduction efficiency.[98]

Conclusions

We have reviewed recent advances in the development of improved Ad vectors for stem cells. Ad vectors have advantages over other viral vectors: the high transduction efficiency, the ease of vector preparation, and the transient expression ability. By the Ad vector-mediated introduction of a differentiation master regulator gene, we could control the differentiation of stem cells. These technical advances should greatly facilitate the analysis of gene function in the stem cells as well as the therapeutic applications of gene-modified stem cells.

Abbreviations Used

ES, embryonic stem; mES, mouse ES; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; Ad, adenovirus; CAR, coxsackievirus and adenovirus receptor; Ad5, Ad serotype 5; ITR, inverted terminal repeats; Ad35, Ad serotype 35; AdRGD vector, Ad vector containing the RGD

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354 peptide; Ad K7 vector, Ad vector containing a polylysine
355 stretch; hES, human ES; STAT3, signal transducer and
356 activator of transcription 3; LIF, leukemia inhibitory factor;
357 STAT3F, dominant-negative mutant of STAT3; hMSCs,
358 human MSCs; BMP2, bone morphogenetic protein 2; AdF35,
359 Ad vector containing the Ad35 fiber.

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RESEARCH ARTICLE

Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities

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Adenoviral gene transfer to hematopoietic stem cells (HSCs)/progenitors would provide a new approach to the treatment of hematopoietic diseases and study of the hematopoietic system. We have previously reported that an adenovirus (Ad) vector composed of whole Ad serotype 35 (Ad35), which belongs to subgroup B, shows efficient gene transfer into human bone marrow CD34⁺ cells. However, Ad35 vector-mediated transduction into human HSCs/progenitors has not yet been fully optimized. In the present study, we have systematically examined promoter activity in the context of Ad35 vectors in human bone marrow CD34⁺ cells and primitive CD34⁺ subsets to optimize the transduction efficiency in human hematopoietic stem/progenitor cells. In the first of the transduction experiments, the improved in vitro ligation method was applied to Ad35 vector construction to allow for simple and efficient production of an E1/E3-deleted Ad35 vector. Using this method, we constructed a series of Ad35 vectors encoding the enhanced green fluorescence protein (GFP) under the control of a variety of strong viral and cellular promoters. Of the six types of promoters tested, significantly higher transduction efficiencies were achieved with the human elongation factor 1α promoter (EF1α promoter), the human cytomegalovirus (CMV) immediate-early 1 gene enhancer/β-actin promoter with β-actin intron (CA promoter), and the CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi promoter) in the human CD34⁺ cells and the immature subsets (CD34⁺CD38^low/−^ and CD34⁺AC133⁺ subsets). In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34⁺ cells and the immature hematopoietic subsets. Furthermore, the CA promoter-mediated GFP-expressing cells differentiated into progenitor cells of all lineages. These results indicate the construction of an optimized Ad35 vector backbone for efficient transduction into HSCs/progenitors.
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Keywords: *adenovirus serotype 35 vector; CD34⁺ cells; CA promoter; hematopoietic stem cells/progenitors*

Introduction

Hematopoietic stem cells (HSCs) have the potential for self-renewal and multilineage differentiation into all mature blood cells. Hence, efficient transduction into HSCs would afford the opportunity to treat a number of diseases that result from abnormal blood cell function, and would be a powerful tool for study of the regulation of proliferation, differentiation, and trafficking of HSCs. For gene transfer into HSCs, moloney-derived retrovirus vectors and lentivirus vectors are often used, although the transduction efficiencies of the retrovirus vectors are disappointingly low in immature HSC/progenitors, probably due to the quiescent state of HSCs and the lack of suitable receptors for vector binding.[1,2] Lentivirus vectors have recently shown promise,[3,4] but their safety remains to be established.

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Among the various types of vectors, adenovirus (Ad) vectors have been widely used for delivery of foreign genes in not only experimental studies but also clinical trials. Advantages which make Ad vectors an attractive vehicle for gene transfer include the ability to easily prepare high-titer stocks of purified vectors, efficient escape from the endosome, and the ability to transport their DNA genome into the nucleus, allowing for efficient transduction in quiescent cells. However, the utility of commonly used Ad vectors, which are based on Ad serotype 2 (Ad2) or Ad serotype 5 (Ad5) belonging to subgroup C, for transduction into human CD34⁺ cells has been limited[5,6] because a primary receptor for Ad2 and Ad5, coxsackievirus and adenovirus receptor (CAR), and second receptors, $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins, are not expressed at sufficient levels in human CD34⁺ cells.[5,7,8] In contrast, it has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34⁺ cells and hematopoietic cell lines.[5,9] We have therefore developed a novel Ad vector, Ad35 vector, which is composed of whole Ad35, and have demonstrated that Ad35 vectors achieve higher levels of transduction efficiency without significant

toxicity in human bone marrow CD34+ cells than both conventional Ad5 vectors and chimeric Ad5F35 vectors, which are fiber-substituted Ad5 vectors containing Ad35 fiber proteins.[10] Ad35 recognizes CD46 (membrane cofactor protein) as a cellular receptor,[11,12] and CD46 is ubiquitously expressed in almost all human cells except for erythrocytes,[13,14] including human cord blood CD34+ cells.[15] Therefore, human CD34+ cells would be considered to be a suitable target for Ad35 vectors.

In addition to receptor expression in target cells, the choice of promoters that drive expression of introduced genes is another crucial determinant for transduction efficiency. Optimization of the promoter leads not only to increased transgene expression but also to decreased vector dose and side effects. A variety of promoters have been used for transduction into human CD34+ cells, including the phosphoglycerate kinase 1 promoter (PGK promoter),[16] the human cytomegalovirus immediate-early region promoter/enhancer (CMV promoter),[17,18] and the CMV immediate-early enhancer/the chicken β-actin promoter with the β-actin intron sequence (CA promoter).[8] However, few studies have simultaneously compared the relative strength of various types of promoters in human CD34+ cells,[19] and information regarding promoter activities in human CD34+ cells is controversial. In addition, the promoter activities have not been fully evaluated in immature CD34+ subpopulations. It is well known that human CD34+ cells are morphologically and functionally heterogeneous and that HSCs/progenitors constitute only a fraction of all CD34+ cells. It is of great importance to evaluate the transduction efficiencies in immature hematopoietic subpopulations.

In the present study, we first applied the improved *in vitro* ligation method developed by Mizuguchi and Kay[20,21] to Ad35 vector construction to facilitate the generation of Ad35 vectors. Second, promoter activities in the context of Ad35 vectors were systematically evaluated in whole human bone marrow CD34+ cells and immature CD34+ subpopulations (CD34+CD38low/- and CD34+AC133+ subsets) to optimize Ad35 vector-mediated transduction into human hematopoietic stem/progenitors. Finally, the proliferative and differentiation potential of the Ad35 vector-mediated transduced CD34+ cells was examined by a colony-forming assay.

Results

Construction of E1/E3-deleted Ad35 vectors by the improved in vitro ligation method

To construct E1/E3-deleted Ad35 vectors simply and efficiently, the improved *in vitro* ligation method was applied to the construction of Ad35 vectors. This method, which was developed by Mizuguchi and Kay,[20,21] is a simple and efficient method by which conventional Ad5 vectors can be constructed. First, all of the E1a region and most of the E1b region were deleted to make the Ad35 vectors replication-incompetent. Then, the three unique restriction sites (I-*CeuI*, *SwaI*, and PI-*SceI*) were introduced into the E1 deletion site to efficiently insert foreign genes into the E1 deletion site of the vector plasmid by a single *in vitro* ligation. The I-*CeuI* and PI-*SceI* sites were used for insertion of foreign genes, while the *SwaI* site was used to reduce the generation of parental, nonrecombinant plasmid. Next, to increase the packaging capacity of the Ad35 vector genome, most of the E3a and E3b regions were deleted. The resulting vector plasmids, pAdMS2, pAdMS3, and pAdMS4, contain the complete Ad35 genome minus the E1 (pAdMS2) or E3 region (pAdMS3) or E1/E3 region (pAdMS4) (Figure 1). Approximately 3 and 1.9 kb of the E1 and E3 regions, respectively, were deleted in pAdMS4.

The Ad35 vector plasmids containing a green fluorescence protein (GFP) gene under the control of a variety of promoters were produced by *in vitro* ligation of I-*CeuI*/PI-*SceI*-digested pAdMS4 and the shuttle plasmids containing a GFP expression cassette. SbfI-linearized Ad35 vector plasmids were transfected into VK10-9 cells (293 cells expressing Ad5 E4 proteins as well as E1 proteins),[22] and the cells were cultured for 10–14 days to produce recombinant Ad35 vectors, followed by the routine method for Ad5 vector preparation. VK10-9 cells can support the replication of Ad35 vectors; however, Ad35 vectors can not grow on 293 cells, as described previously.[10] The final yields of the Ad35 vectors were equivalent to those described previously.[10,23]

Relationship between CD46 expression and transduction efficiency in human bone marrow CD34+ cells

Recently, the complement regulatory protein CD46 has been identified as a cellular receptor for Ad subgroup B.[11,12] To study the role of CD46 in Ad35 vector-mediated transduction into human bone marrow CD34+ cells, we evaluated the relationship between CD46 expression levels and the transduction efficiencies of Ad35 vector containing the CMV promoter. The CMV promoter is generally regarded as one of the most powerful

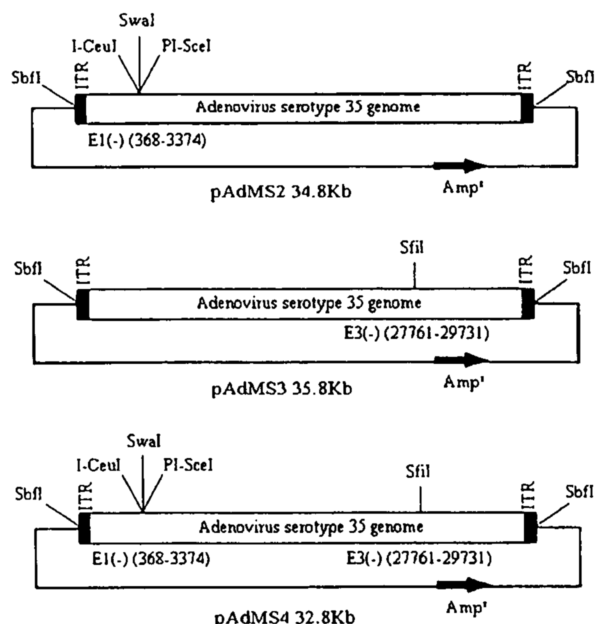

Figure 1 Structure of the vector plasmids pAdMS2, -3, and -4 for construction of Ad35 vectors by the improved in vitro ligation method.

promoters[24] and is widely used in transduction experiments. As shown in Figure 2a, almost all the bone marrow CD34+ cells expressed high levels of CD46, similar to the cord blood CD34+ cells.[15] Significant amounts of CD46 were detected in all the CD34+ cells from three different donors (data not shown). In the transduction experiments using Ad35 vector containing the CMV promoter, the percentage of GFP-positive cells was proportional to the vector concentration at the lower vector doses of 1000–6000 VP/cell (Figure 2b). However, despite the high levels of CD46 expression, the transduction efficiencies of the Ad35 vector did not correlate with the vector concentrations at the higher vector dose reaching a plateau at more than 12 000 VP/cell. There was no correlation between the levels of CD46 expression and the GFP expression levels (Figure 2c). These data indicate that factors other than the CD46 expression levels also determine the transduction efficiencies of Ad35 vectors in human bone marrow CD34+ cells.

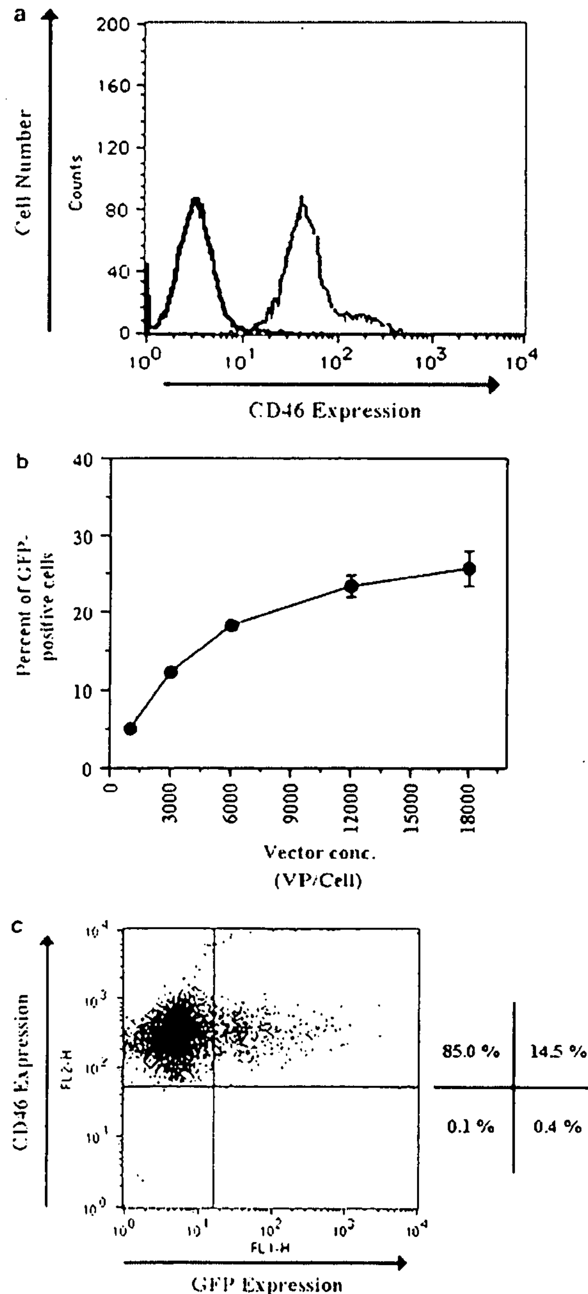

Figure 2 (a) CD46 expression in human bone marrow CD34+ cells. The cells were incubated with FITC-conjugated anti-CD46 antibody. As a negative control, the cells were incubated with an irrelevant antibody (shaded histogram). Similar levels of CD46 were found in the cells from three different donors. (b) Dose-response of the percentage of GFP-positive cells following transduction with Ad35 vector containing a CMV promoter-driven GFP expression cassette. Human bone marrow CD34+ cells were transduced with the Ad35 vector at the indicated vector concentrations for 6 h, washed, and resuspended in medium. After 48 h later, GFP expression was measured by flow cytometry. All data represent the means ± s.d. of three experiments. (c) Relationship between the CD46 expression level on human bone marrow CD34+ cells and GFP expression levels following Ad35 vector transduction. The cells were transduced with the Ad35 vector containing the CMV promoter at 6000 VP/cell for 6 h, washed and resuspended in the medium. After 48 h of incubation and washing, the transduced cells were incubated with an anti-CD46 antibody. The cells were then washed, resuspended, and incubated with PE-conjugated second antibody. The percentage of stained cells found in each quadrant is indicated. Data shown are from one representative experiment of the three performed.

Transduction with Ad35 vectors containing various types of promoters in human bone marrow CD34+ cells

The refractoriness of the human CD34+ cells to Ad35 vector-mediated transduction might be due to promoters that drive expression of a foreign gene. To examine the promoter activities in the CD34+ cells, we investigated the transduction efficiencies of the Ad35 vectors containing various types of promoters in the CD34+ cells at 6000 VP/cell. The following promoters were tested: the CMV promoter, the EF1α promoter, the CA promoter, the mouse PGK promoter, the murine stem cell virus (MSCV) long terminal repeat (LTR) promoter (MSCV promoter), and the CMV promoter/enhancer containing the largest intron of CMV (intron A) (CMVi promoter). Of the six types of promoters, the CA, EF1α, and CMVi promoters were found to allow higher levels of GFP expression than the CMV, PGK, and MSCV promoters (Figure 3). In particular, the highest percentage of GFP-positive cells was obtained with the CA promoter (53.8%). The relative promoter strength in terms of percentage of GFP-positive cells was the CA (53.8%) > CMVi (44.0%) > EF1α (40.9%) > PGK (23.7%) > CMV (20.3%) > MSCV (10.5%). The mean fluorescence intensity (MFI) was also more than 3.5-fold higher with the CA and CMVi promoters than with the other types of promoters. Continuous incubation of the CD34+ cells with Ad35 vector containing the CA promoter at 6000 VP/cell for 48 h led to an increase in GFP-positive cells up to 67% (data not shown). These data indicate that the transduction efficiencies in human bone marrow CD34+ cells are largely dependent on the promoter and that the CA promoter is the most active in CD34+ cells among the six types of promoters examined in the present study.

Next, to determine whether the Ad35 vectors had infected the CD34+ cells that did not express GFP, the

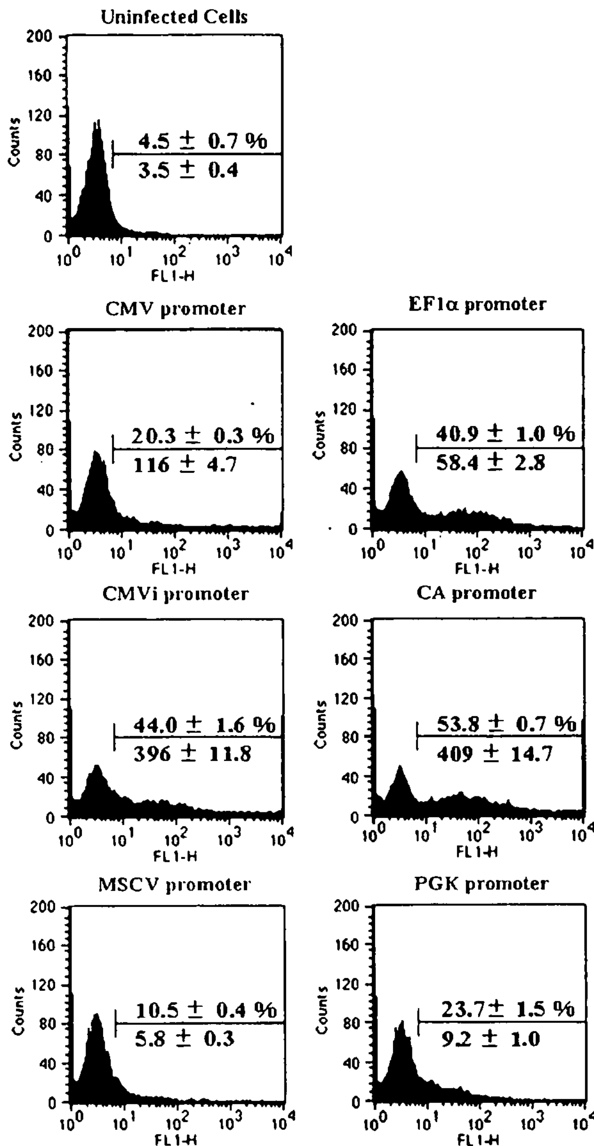

Figure 3 Comparison of promoter activities in human bone marrow CD34⁺ cells transduced with Ad35 vectors. The results are shown as a percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The CD34⁺ cells were transduced with Ad35 vectors at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means±s.d. of three experiments.

amounts of intracellular vector genomes in the GFP-positive and -negative fractions were measured by real-time PCR. As CD34⁺ cells are functionally and morphologically heterogeneous, the promoters may not be active in all CD34⁺ cells. Real-time PCR analysis demonstrated that approximately 300 copies of the Ad35 vector genomes per β-actin copy were detected in both GFP-positive and -negative fractions following transduction with the Ad35 vectors containing any type of promoter (Figure 4). These results suggest that Ad35 vectors would infect all CD34⁺ cells, probably via CD46; however, not all infected cells express GFP.

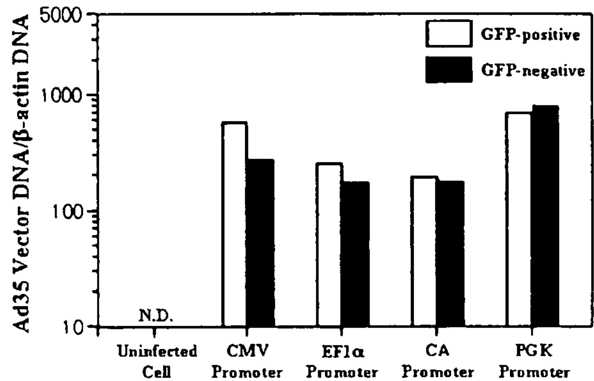

Figure 4 Ad35 vector copy numbers in GFP-positive and -negative cells following Ad35 vector transduction into human bone marrow CD34⁺ cells. The CD34⁺ cells were transduced with Ad35 vectors at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP-positive and -negative cells were sorted and the total DNA was extracted from the cells. The copy numbers of Ad35 vectors and β-actin were analyzed by Taqman PCR. All data represent the means of two independent experiments. ND, not detected (under the limit of detection).

Transduction in immature subpopulations of human bone marrow CD34⁺ cells

Next, to examine the promoter activities in the primitive hematopoietic subpopulations among the CD34⁺ cells, CD34⁺CD38$^{low/-}$ cells and CD34⁺AC133⁺ cells were transduced with the Ad35 vectors. CD34⁺CD38$^{low/-}$ and CD34⁺AC133⁺ cells are known to be the more primitive subsets among the CD34⁺ cells.[25–27] Transduction experiments demonstrated that the CA, EF1α, and CMVi promoters were clearly superior in CD34⁺CD38$^{low/-}$ subsets compared with the CMV, MSCV, and PGK promoters (Figure 5). Similar results were obtained for CD34⁺AC133⁺ subsets (Figure 6). Among these three promoters, use of the CA promoters resulted in the highest transgene expression in both CD34⁺CD38$^{low/-}$ and CD34⁺AC133⁺ subsets (57% GFP-positive for CD34⁺CD38$^{low/-}$ subsets, 51% GFP-positive for CD34⁺AC133⁺ subsets). These data indicate that the CA, EF1α, and CMVi promoters mediate the higher transduction efficiencies and that the CA promoter is the most efficient in these immature CD34⁺ subpopulations.

CA promoter activity in the colony-forming hematopoietic progenitors

To evaluate the gene expression potential of the CA promoter in colony-forming hematopoietic progenitors, the transduced cells were sorted into GFP-positive and -negative cells following transduction with the Ad35 vector containing the CA promoter, and colony-forming assays were then performed for the sorted GFP-positive and -negative cells. Values indicating the colony-forming unit (CFU) content of the sorted cells 14 days after plating are shown in Table 1. Comparing the total number of colonies derived from the GFP-positive cells with those from the uninfected cells and the GFP-negative cells, it appears that the GFP-positive cells formed almost the same total numbers of colonies as the uninfected cells and the GFP-negative cells. CFU-granulocyte-macrophage (CFU-GM) colonies were grown without significant reduction from the CA promoter-mediated GFP-positive cells, compared with

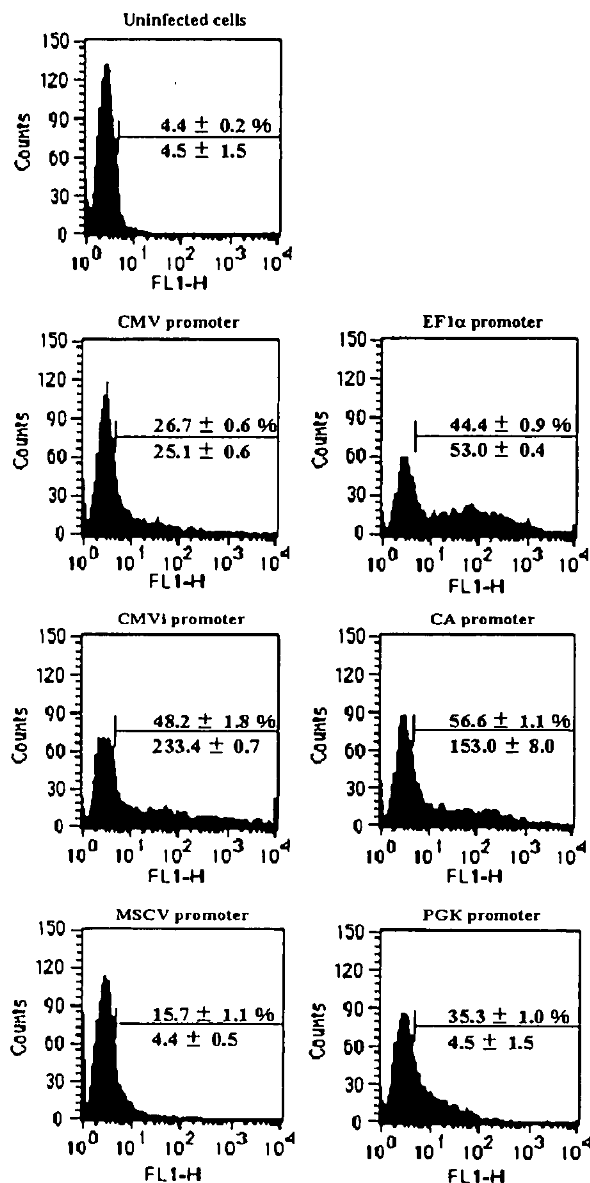

Figure 5 Comparison of promoter activities in the $CD34^+CD38^{low/-}$ subsets transduced with Ad35 vectors. Results are shown as the percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The $CD34^+CD38^{low/-}$ subsets were transduced at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means±s.d. of two experiments.

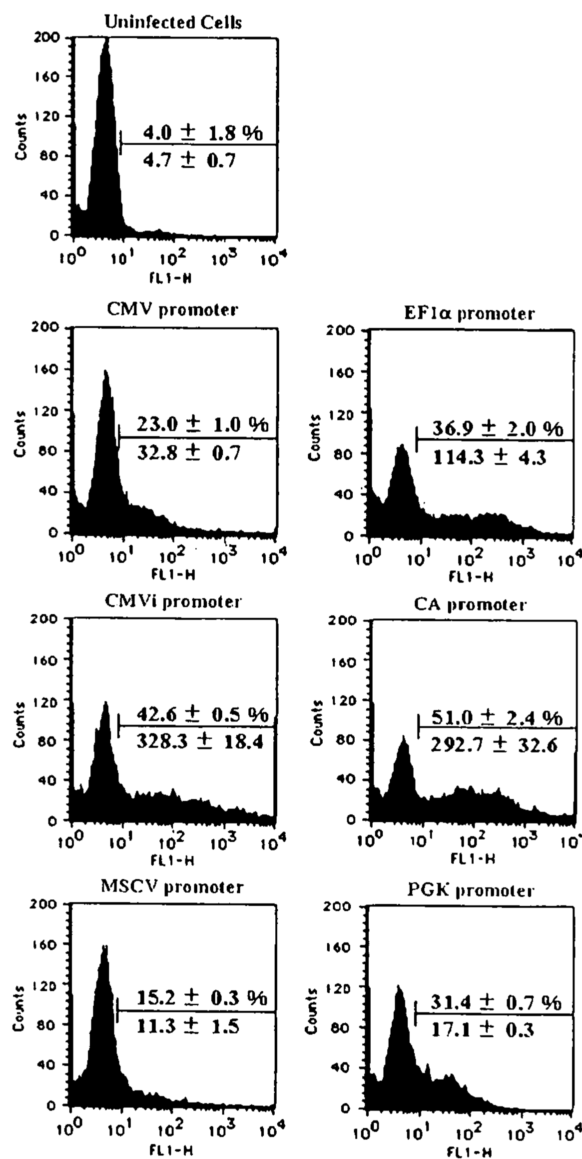

Figure 6 Comparison of promoter activities in human bone marrow $CD34^+AC133^+$ subsets transduced with Ad35 vectors. Results are shown as the percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The $CD34^+AC133^+$ subsets were transduced at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means±s.d. of two experiments.

the GFP-negative cells, although the growth of burst-forming units-erythroid (BFU-E) colonies was slightly impaired in the GFP-positive cells. CFU-granulocyte erythrocyte monocyte macrophage (CFU-Mix) colonies, which are derived from the most primitive hematopoietic progenitors, were also found in GFP-positive cells. These data suggest that the CA promoter would be significantly active in immature colony-forming progenitors. However, the size of colonies from both GFP-positive and -negative cells appeared to be smaller than that in uninfected cells (data not shown), suggesting that exposure to Ad35 vectors slightly impairs the growth of colony-forming bone marrow hematopoietic progenitors under these conditions.

Discussion

The choice of a promoter to drive transgene expression is important in gene transfer experiments. Currently, a few systematic examinations of promoter activities have been carried out in gene transfer experiments *in vivo*[28–30] and

Table 1 Numbers of colonies derived from GFP-positive and -negative cells following transduction with the Ad35 vector containing the CA promoter in human CD34⁺ cells

CD34⁺ cells	*Total*	*BFU-E*	*CFU-GM*	*CFU-Mix*
Sample 1				
Uninfected cells	222.8±25.5	72±11.8	150.3±14.2	0.5±0.6
GFP-positive	193.5±29 (86.9%)	61±9.9 (84.7%)	131.5±17.7 (87.5%)	1±1.4
GFP-negative	180.5±13.4 (81%)	24.5±0.7 (34%)	155.5±13.4 (103.5%)	0.5±0.7
Sample 2				
Uninfected cells	124.8±13.5	44.5±6.1	78.8±9.5	1.5±0.6
GFP-positive	115±11.3 (92%)	29±5.7 (65.2%)	85.5±6.4 (108.5%)	0.5±0.7
GFP-negative	158±19.8 (127%)	26±0 (58.4%)	130.5±20.5 (165.6%)	1.5±0.7

The data represent the mean number of colonies±s.d. from duplicate cultures and the percentage of number of colonies/uninfected cells.

in vitro;[19,29,31] however, information regarding the promoter activities in HSCs is limited and controversial. The aim of this study was to identify an Ad35 vector platform for efficient transgene expression in human HSCs by optimizing a promoter that directs transgene expression. For this purpose, we constructed a series of Ad35 vectors in which GFP expression was driven by a variety of promoters and compared the levels of GFP expression in human bone marrow CD34⁺ cells and immature CD34⁺ subsets.

In the present study, we examined the following promoter activities in human bone marrow CD34⁺ cells: the CMV, EF1α, CMVi, CA, MSCV, and PGK promoters, which are widely used in transduction experiments. Comparison of the six types of promoters showed that a significant increase in GFP-positive cells was obtained with the EF1α, CMVi, and CA promoters. Among these three promoters, the CA promoter was the most efficient at transducing human bone marrow CD34⁺ cells (Figure 3) and immature CD34⁺ subsets (CD34⁺CD38^low/−^ and CD34⁺AC133⁺ subsets) (Figures 5 and 6) under the condition employed. Furthermore, the CA promoter-mediated GFP-positive cells formed nearly the same numbers of colonies as uninfected cells in the colony assay (Table 1). The powerful activity of the CA promoter has been demonstrated in important target cells for gene therapy, including dendritic cells,[32] lymphocytes,[32] and hepatocytes.[33] In addition, earlier work with the CA promoter noted heightened transgene expression in immature cells. Okabe *et al*[34] have demonstrated that the CA promoter functions in the embryos of transgenic mice. Efficient transgene expression has also been achieved with the CA promoter in murine embryonic stem cells.[35,36] The data described above indicate that the CA promoter would be the promoter of choice for high levels of transgene expression in immature cells, including HSCs/progenitors.

The CMV promoter is one of the strongest promoters and is currently in wide use for transient gene expression experiments. However, the CMV promoter did not mediate high levels of GFP expression in the CD34⁺ cells (Figure 2b), immature subsets (Figures 5 and 6), and colony-forming CD34⁺ progenitors (data not shown). Several groups have demonstrated that the CMV promoter does not allow for high transduction efficiencies in human CD34⁺ cells[3,19] and murine embryonic stem cells,[36,37] suggesting that the CMV promoter might not be appropriate for immature cells. However, the inclusion of intron A into the CMV promoter (CMVi promoter) largely increases GFP expression in the CD34⁺ cells as well as immature CD34⁺ subsets. The β-actin intron is also included in the CA promoter. These data suggest that an intron may be a key element for efficient transgene expression in human CD34⁺ cells, although a detailed mechanism for enhancement of transgene expression by intron A in CD34⁺ cells has not yet been elucidated.

Recently, the human membrane cofactor protein CD46 has been shown to be a cellular receptor for subgroup B Ad, including Ad35.[11,12] CD46 is a single-chain type I transmembrane glycoprotein that is expressed in almost all human cells.[13,14] The ubiquitous expression of CD46 leads to a broad tropism of Ad35 vectors toward human cultured cells compared with Ad5 vectors.[23] Expression levels of CD46 on the cells appear to correlate with the affinity of Ad35 for the cells, which is similar to the relationship between CAR expression levels on the cells and the transduction efficiencies of Ad5 vectors, as reported below. Segerman *et al*[9] have demonstrated almost 100% binding of Ad35 to human hematopoietic cell lines, Jurkat, K562, and HL-60 cells, which express sufficient levels of CD46.[38,39] The transduction efficiencies of the chimeric Ad5F35 vector, which is an Ad5-based vector containing an Ad35 fiber shaft and knob, have been found to increase progressively with CD46 expression density on a panel of CHO cells stably expressing CD46.[40] Shayakhmetov *et al*[5] have reported the efficient attachment and internalization of Ad35 in human bone marrow CD34⁺ cells, which express high level of CD46 (Figure 2a). In this study, however, the transduction efficiencies in the CD34⁺ cells unexpectedly did not increase proportionally with the increased dose of Ad35 vectors, despite the high expression levels of CD46 (Figure 2b). Even the CA promoter, which was the most efficient in this study, did not mediate more than 70% of GFP-positive cells at the increased dose (data not shown). The limitation of the transduction efficiencies in the CD34⁺ cells is likely due to the promoter activities that direct transgene expression. We have demonstrated that similar amounts of Ad35 vector genome could be detected in both GFP-positive and -negative cells (Figure 4). These results suggest that Ad35 vectors would interact with all of the CD34⁺ cells via CD46; however, the promoters examined here do not function in all infected CD34⁺ cells. It is well known that human CD34⁺ cells are heterogeneous. With the GFP-negative cells

that were infected by the Ad35 vectors, there may not be sufficient enough levels of the transcriptional factors for promoter function. Transduction with Ad35 vector containing a more suitable promoter might result in much higher transduction efficiencies in human CD34⁺ cells. A similar phenomenon was observed in the study by Shayakhmetov *et al*,[5] in which human CD34⁺ cells were transduced with the chimeric Ad5F35 vector. The genome of the chimeric Ad5 vector was detected in both GFP-positive and -negative cells.

Segerman *et al*[41] demonstrated that there are two different receptors for Ad35 in human cells. One is CD46 and another receptor is currently unidentified. It remains to be clarified whether human CD34⁺ cells express the unidentified receptor for Ad35 and whether the unknown receptor plays an important role on Ad35 vector-mediated transduction in the CD34⁺ cells.

A number of studies have evaluated the transduction efficiencies in CD34⁺CD38$^{low/-}$ cells by staining the transduced CD34⁺ cells with anti-CD38 antibody just before flow-cytometric analysis, not before transduction.[6,42,43] However, in this study, sorted CD34⁺CD38$^{low/-}$ and CD34⁺AC133⁺ subpopulations were transduced with Ad35 vectors to evaluate the transduction efficiencies in these immature CD34⁺ subsets because the expression levels of CD38 in the CD34⁺ cells would decrease during culture, irrespective of the mature/immature state. Dorrell *et al*[44] and Donaldson *et al*[45] have reported that a dramatic increase in CD34⁺CD38 cell frequency occurred during culture; however, these cells lost the potential to repopulate in the NOD/SCID mouse and to form colonies in the colony-forming assay, respectively. We confirmed that the expression levels of CD38 and AC133 decreased after a 2-day culture (data not shown). As such, the CD34⁺ cells might have to be sorted before transduction to truly evaluate the transduction efficiencies in primitive hematopoietic subsets. In addition, because the CD34⁺ cells may differentiate during culture and Ad35 vectors may infect differentiated cells efficiently, Ad35 vectors in the present study were incubated with the CD34⁺ cells for 6 h and the cells were then washed to remove Ad35 vectors. More efficient transduction with Ad35 vectors was achieved when the CD34⁺ cells were continuously cultured with Ad35 vectors for 48 h (data not shown), as reported previously.[10]

Various methods have been established for feasible generation of Ad vectors.[46] Among these methods, the improved *in vitro* ligation method, which was developed by Mizuguchi and Kay,[20,21] is a simple and efficient method based on plasmid construction. To efficiently construct a series of Ad35 vectors, an improved *in vitro* ligation system was applied to construction of replication-incompetent E1/E3-deleted Ad35 vectors. We have previously constructed Ad35 vector plasmids by gel purification of DNA fragments and two-step *in vitro* ligation.[10] Reddy *et al*[47] have also constructed Ad35 vector plasmids by several steps of *in vitro* ligation of DNA fragments. Vogels *et al*[48] and Gao *et al*[49] have reported construction of recombinant Ad35 vectors by homologous recombination in 293-derived cell lines and PER.C6 cells stably expressing Ad35 E1B 55K, respectively. All of these methods described above are time-consuming and inefficient. Using the improved *in vitro* ligation method, we more rapidly obtained yields of Ad35 vectors similar to those reported previously.[10] In addition, based on the sequence information of the Ad35 E1 and E3 regions, the E1 deletion size has been increased and most of the E3 region has been deleted in pAdMS4, leading to an increase in the packaging capacity. The increase in the deletion size in the E1 and E3 regions did not reduce the transduction efficiencies of Ad35 vectors (data not shown). Further deletions in the E1 and E3 coding regions must make it possible to insert larger foreign genes into the Ad35 vectors.

As the Ad genome does not integrate into the host genome, transgene expression via Ad vectors can occurr transiently, which is suitable for *ex vivo* manipulation of HSCs and the study of gene functions. This property also results in a low risk of insertion mutagenesis, but stable transgene expression is not allowed. To address this limitation, several groups have developed hybrid Ad vectors such as Ad/adeno-associated virus vectors, which can integrate viral genome into the host genome.[50–52] The Ad35 vector would be a promising framework for the development of these improved vectors.

In summary, we have demonstrated that the EF1α, CMV promoter containing intron A, and the hybrid CA promoter is superior at transducing human bone marrow CD34⁺ cells in the context of Ad35 vector. In particular, the CA promoter functions most efficiently in CD34⁺ cells and immature CD34⁺ subsets. The results of our study provide valuable information regarding gene transfer into HSCs.

Materials and methods

Plasmids

Vector plasmids pAdMS2, -3, and -4 were constructed as follows. The *Sbf*I/*Pst*I fragment of pFS2-Ad35-7,[10] which has the left end of the Ad35 genome (bp 1–367 and 2917–3670) with an E1 deletion, was ligated with the *Sbf*I site of pFS2,[10] resulting in pFS2-Ad35-9. (The end of the *Sbf*I site is compatible with a *Pst*I site.) pFS2-Ad35-9 was cut by *Pac*I and *Bam*HI, and ligated with oligonucleotides 1 (5′-TATAACTATAACGGTCCTAAGGTAGCGA*ATTTAAAT* **ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCA**-3′) and 2 (5′-GATC**TGCCATTTCATTACCTCTTTCTCCGCACCCGACATAGAT** *ATTTAAAT*TCGCTACCTTAGGACCGTTATAGTTATAAT-3′) (I-*Ceu*I, *Swa*I, and PI-*Sce*I recognition sequences are noted by underlining, italics, and bold, respectively), resulting in pFS2-Ad35-10, which contains I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion site of the Ad35 genome. The *Sbf*I/*Pst*I fragments of pFS2-Ad35-7 and pFS2-Ad35-10 were then ligated, resulting in pFS2-Ad35-11. The *Sbf*I/*Asc*I fragment of pFS2-Ad35-11 was exchanged with the *Sbf*I/*Asc*I fragment of pAdMS1,[10] which clones the whole Ad35 genome, resulting in pAdMS2-1. The *Not*I site of pAdMS2-1 was changed into an *Sbf*I site by using oligonucleotide 3 (5′-GGCCCCTGCAGG-3′) (the *Sbf*I recognition sequence is underlined), resulting in pAdMS2. To delete the E3 region in the Ad35 genome, the *Bam*HI/*Not*I fragment of pHM15-Ad35-1,[10] which has the right end of the Ad35 genome (bp 29732 – right end of the genome), was cloned into *Bam*HI/*Not*I sites of a shuttle plasmid pFS1, which contains multicloning sites composed of *Sal*I/*Sbf*I/*Eco*RI/*Bam*HI/*Swa*I/*Not*I sites, creating pFS1-Ad35-1. pFS1 was constructed by ligation of *Xba*I/*Sac*I-digested pGEM7Zf(+) (Promega

Corp., MA, USA) with the oligonucleotides containing the multi-cloning sites. The *SalI/EcoRI* fragment of pHM15-A35-1 (bp 23583-27760) was inserted between the *SalI/EcoRI* sites of pFS1-Ad35-1, resulting in pFS1-Ad35-2. pFS1-Ad35-2 was then cut by *EcoRI/BamHI* and ligated with oligonuclotide 4 (5′-AATTGGCCACGTAGGCC-3′) and 5 (5′-GATCGGCCTACGTGGCC-3′) (*SfiI* recognition sequence is underlined), resulting in pFS1-Ad35-9. The *SalI/NotI* fragment of pFS1-Ad35-9 was ligated with the *SalI/NotI* fragment of pHM14-Ad35-1, creating pHM14-Ad35-3. pHM14-Ad35-1 was constructed by cloning of the *EcoRI/KpnI* fragment (bp 21945–29545) of the Ad35 genome into the *EcoRI/KpnI* sites of pHM14.[53] The *EcoRI/NotI* fragment of pHM14-Ad35-3 was ligated with the *EcoRI/NotI* fragment of pAdMS1, resulting in pAdMS3-1. The *NotI* site of pAdMS3-1 was changed into an *SbfI* site by using oligonucleotide 3, resulting in pAdMS3. The *SbfI/AscI* fragment of pFS2-Ad35-11 was ligated with the *SbfI/AscI* fragment of pAdMS3-1, creating pAdMS4-1. The *NotI* site of pAdMS4-1 was changed into a *SbfI* site by using oligonucleotide 3, resulting in pAdMS4. pAdMS2 and -4 have I-*CeuI*, *SwaI*, and PI-*SceI* sites in the E1 deletion region (ΔE1: bp 368–3374). pAdMS3 and -4 have an *SfiI* site in the E3 deletion region (ΔE3: bp 27 761–29 731). The E1a and E1b coding regions of Ad35 are located from bp 569 to 1441 and from bp 1611 to 3400, respectively, according to the Ad35 genome sequence (GenBank Accession No. AY271307). The E3a and E3b coding regions of Ad35 are located from bp 27 199 to 29 496 and from bp 29 538 to 30 622, respectively. pAdMS2, -3, and -4 have *SbfI* sites at both ends of the Ad genome.

Shuttle plasmids containing a variety of promoters were constructed by changing the CMV promoter of pHMCMV5[21] into another type of promoter, including the EF1α promoter, the CA promoter, the mouse PGK promoter, the MSCV promoter, and the CMVi promoter. The EF1α promoter is derived from pEF1α/myc/nuc (Invitrogen, Carlsbad, CA, USA). The CMVi promoter is derived from pGeneGrip (Gene Therapy Systems, San Diego, CA, USA). The composite CA promoter and PGK promoter were kindly provided by Dr J Miyazaki (Osaka University, Osaka, Japan) and Dr MA Kay (Standford University, CA, USA), respectively. The MSCV promoter was a kind gift of Dr RG Hawley (American Red Cross, MD, USA).

E1/E3-deleted Ad35 vectors expressing enhanced GFP

To construct the plasmid for a recombinant E1/E3-deleted Ad35 vector containing a CMV promoter-driven GFP expression cassette, pHMCMV-GFP1[54] and pAdMS4 were digested with I-*CeuI* and PI-*SceI*. The digested pAdMS4 was ligated with the I-*CeuI*/PI-*SceI* fragment of pHMCMV-GFP1 containing a GFP expression cassette, resulting in pAdMS4-CMVGFP. pAdMS4-CMVGFP was linearized by the digestion with *SbfI*. The linearized DNA was transfected into VK10-9 cells (kindly provided by Dr V Krougliak),[22] which are 293 cells expressing the E4 proteins of Ad5 as well as the E1 proteins. A cytopathic effect (CPE) was observed 10–14 days after transfection, and the virus was then amplified in VK10-9 cells and purified by the conventional method for Ad5 vector preparation. For the preparation of recombinant E1/E3-deleted Ad35 vectors containing various types of promoters, a *GFP* gene was cloned into multi-cloning sites in the shuttle plasmids containing various types of promoters, and the Ad35 vectors were then prepared by methods similar to those described above.

Transduction experiment

Human bone marrow CD34⁺ cells were purchased from Biowhittaker, Inc., Walkersville, MD, USA. The cells were recovered from the frozen stock, suspended in StemSpan™ 2000 containing cytokine cocktail StemSpan™ CC100 (human Flt-3 ligand (100 ng/ml), human stem cell factor (100 ng/ml), human interleukin (IL)-3 (20 ng/ml), and human IL-6 (20 ng/ml)) (StemCell Technologies Inc., Vancouver, BC, Canada), and were seeded into a 48- or 96-well plate ($1–5 \times 10^4$ cells/well). The cells were transduced with the GFP-expressing Ad35 vectors at the indicated VP/cell 16–18 h after seeding. At 6 h after incubation, the cells were washed to remove the Ad35 vectors and resuspended in the medium. At 48 h after transduction, 10^4 cells per sample were analyzed for GFP expression by flow cytometry on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Tokyo, Japan).

Flow-cytometric analysis of CD46 expression

Human bone marrow CD34⁺ cells were suspended in staining buffer containing fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD46 antibody (Pharmingen, San Diego, CA, USA). After washing with the sorting solution, the stained cells (10^4 cells) were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson). For simultaneous analysis of GFP and CD46 expression, the transduced cells were incubated with mouse anti-human CD46 antibody (Pharmingen). Subsequently, the cells were washed and incubated with phycoerythrin (PE)-conjugated goat anti-mouse IgG second antibody (Pharmingen). After washing with the sorting solution, the analysis was performed as described above.

Real-time quantitative PCR

Human bone marrow CD34⁺ cells were incubated with the Ad35 vectors at 6000 VP/cell, and control cells were incubated without the Ad35 vectors. After a 6-h incubation, the medium was changed to remove the Ad35 vectors. At 48 h after transduction, the cells were harvested, pelleted, and washed gently. The cells were then sorted into GFP-positive and -negative fractions using a FACSVantage SE (Becton Dickinson). Sort purities were greater than 90% for both GFP-positive and -negative fractions. The sorted cells were treated with trypsin and DNase, followed by washing to remove the extracellular vector genome. Total DNA, including the Ad35 vector DNA, was extracted from the GFP-positive and -negative cells using a Tissue DNeasy Kit (Qiagen, Valencia, CA, USA). The quantitative real-time PCR was performed with 2.5 ng of sample DNA, 0.5 μM each primer, 0.16 μM TaqMan probe, and 25 μl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 50 μl using the ABI Prism 7000 sequence detection system (Applied Biosystems). The PCR was initially denatured at 95°C for 10 min and then subjected to cycles of 95°C for 15 s and

60°C for 1 min. The reaction was carried out for 50 cycles. Primers for amplification were located in the pIX region of Ad35 genome. The sequences of the primers and probe used were as follows: forward, 5′-TGGATGGAAG ACCCGTTCAA-3′; reverse, 5′-CGTCCAAAGGTGAAG AACTTAAAGT-3′; probe, 5′-FAM-CGCCAATTCTTC AACGCTGACCTATGC-TAMRA-3′. These sequences were designed using Primer Express softeware version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired size. The Ad35 vector plasmid pAdMS4 was used as a standard. For human β-actin quantification, β-actin control reagent (Applied Biosystems) was used.

Purification of immature CD34+ subpopulations

Human bone marrow CD34+ cells were incubated with PE-conjugated mouse anti-human AC133 monoclonal antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) or FITC-conjugated mouse anti-human CD38 monoclonal antibody (eBioscience, San Diego, CA, USA) immediately after the cells were recovered from the frozen stock. After washing, cell sorting was performed using a FACSVantage SE (Becton Dickinson). Sorting gates were set to sort the CD38low/− and AC133+ subpopulations. Sort purities were greater than 80% for both CD38low/− and AC133+ subpopulations. The sorted CD34+CD38low/− and CD34+AC133+ cells were transduced with the Ad35 vectors at 6000 VP/cell, as described above.

Colony-forming assay

The GFP-positive and -negative cells were recovered 48 h after transduction with the Ad35 vector containing the CA promoter, as described above. In all, 1000 cells of each fraction were then plated in a 35-mm dish containing Methocult H4444 methylcellulose medium (erythropoietin; 3 U/ml, stem cell factor; 50 ng/ml, GM-CSF; 10 ng/ml, IL-3; 10 ng/ml) (Stem Cell Technologies). After 14 days of incubation at 37°C in a 5% CO_2 incubator, CFU-GM, BFU-E, and CFU-Mix colonies were enumerated under a microscope. The experiments were performed in duplicate. Uninfected cells were also sorted into a GFP-negative fraction and treated as described above.

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Riccardin C: A natural product that functions as a liver X receptor (LXR)α agonist and an LXRβ antagonist

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Abstract Liver X receptors (LXRs) α and β share considerable sequence homology and several functions, respond to the same endogenous and synthetic ligands, and play critical roles in maintaining lipid homeostasis. In this study, liverwort-derived riccardin C (RC) and F (RF) were identified as an LXRα agonist/LXRβ antagonist and an LXRα antagonist, respectively. RC and RF bound to LXRs, but had different abilities to recruit a coactivator and thereby induce transactivation. Despite its unique subtype-selective activity, RC enhanced ABCA1 and ABCG1 expression and cellular cholesterol efflux in THP-1 cells. RC may provide a novel tool for identifying subtype-function and drug development.
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Keywords: Liver X receptor; ATP-binding cassette transporter A1; ATP-binding cassette transporter G1; HDL; Cholesterol

1. Introduction

The liver X receptors α and β (LXRα and LXRβ) are nuclear receptors that form obligate heterodimers with retinoid X receptor (RXR) [1]. LXRs are activated by several oxysterols or intermediates in the cholesterol synthetic pathway [2,3] and serve as key regulators of cholesterol homeostasis by coordinately regulating several genes involved in the efflux, transport, and excretion of cholesterol. These genes include ATP-binding cassette transporter (ABC) A1, ABCG1, and apolipoprotein E – which mediate cellular cholesterol efflux; cholesterol 7α-hydroxylase – the rate liming enzyme for the conversion of cholesterol to bile acids in the liver; and ABCG5/ABCG8–transporters involved in cholesterol /sterol excretion from the liver and intestine [1]. LXR activation also upregulates genes involved in fatty acid and triglyceride synthesis by inducing sterol regulatory element-binding protein-1c (SREBP-1c) – the master regulator of genes involved in lipogenesis [4–6] – and fatty acid synthase expression [7].

LXRα and LXRβ share a high degree of amino acid similarity (78%), have similar binding affinities to physiological oxysterol ligands [2,3], and have several common functions, including the upregulation of ABCA1 and ABCG1 expression [8,9]. However, these receptors have different tissue distributions: while LXRβ is ubiquitously expressed, the expression of LXRα is limited to the liver, kidney, intestine, adipose tissue, and macrophages [10]. In the liver, LXRα serves as an important regulator of cholesterol catabolism. Mice lacking LXRα has no resistance to dietary cholesterol and fail to upregulate hepatic cholesterol 7α-hydroxylase [11,12], whereas LXRβ null mice maintain their resistance. The role of LXR subtypes in triglyceride metabolism is uncertain. One study has shown a reduction in the liver triglyceride level of LXRβ null mice on standard diet [13], whereas a reduction in the expression of lipogenic genes was observed in LXRα null mice fed cholesterol [11,12]. Thus, selective agonists for each LXR subtype may be necessary to elucidate their precise functions.

Riccardin C (RC) and F (RF) are non-sterol natural products isolated from liverworts [14,15] (Fig. 1A). In the present study, we discovered that RC functions as an LXRα-selective agonist/LXRβ antagonist but effectively enhances cholesterol efflux from THP-1 cells.

2. Materials and methods

2.1. Riccardins C and F

RC and RF were purified from a methanol extract of the liverwort *Blasia pusilla* as described previously [14,15].

2.2. Transient transfections and reporter gene assay

CV-1 cells maintained in DMEM containing 10% FCS were co-transfected with 248 ng of LXR response element (LXRE)-driven luciferase vector (pLXREx4-tk-Luc), 248 ng of pSV-β-galactosidase control vector (Promega), and 1.25 ng each of pcDNA3.1-LXRα or LXRβ and pcDNA3.1-RXRα with Polyfect (Qiagen) in 24-well plates according to a previously described method [16]. Three hours after transfection, the cells were treated with test compounds in DMEM containing 10% delipidated FBS, 20 μM compactin, and 10 μM mevalonic acid for 24 h. The cells were then lysed and the reporter gene activity was determined. Luciferase activity was normalized to that of β-galactosidase for each well.

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Abbreviations: LXR, liver X receptor; RXR, retinoid X receptor; ABC, ATP-binding cassette transporter; SREBP-1c, sterol regulatory element-binding protein-1c; LXRE, LXR response element; 22(*R*)HC, 22(*R*)-hydroxycholesterol; TO-1317, TO-901317

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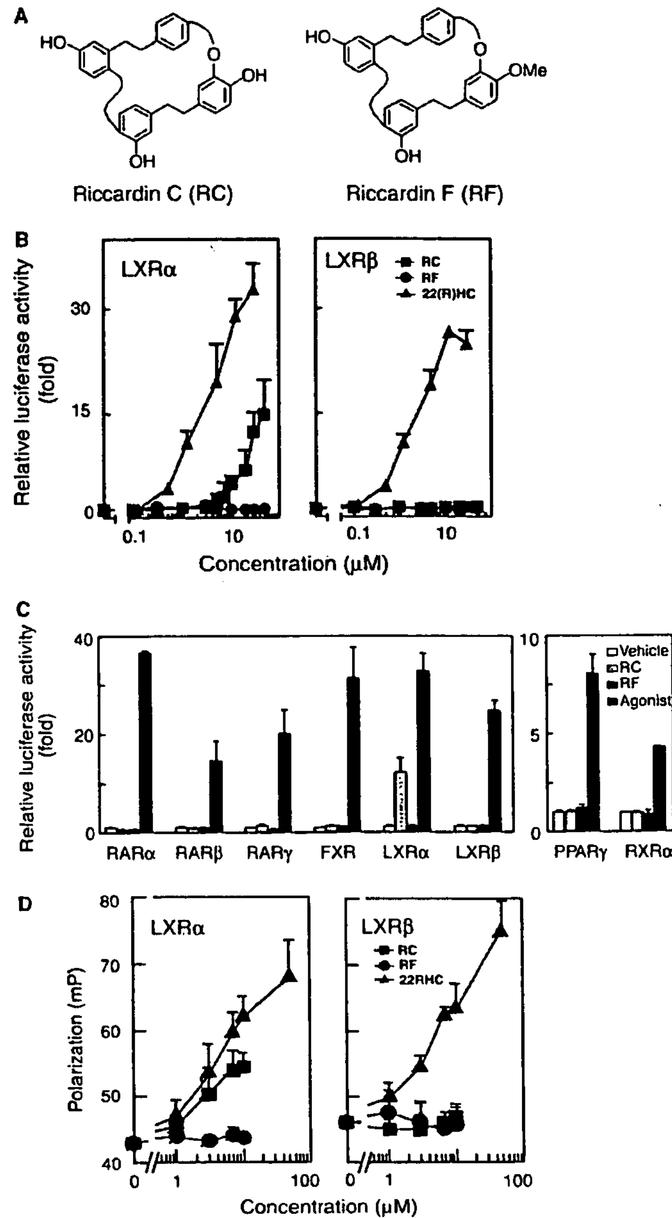

Fig. 1. RC activates LXRα but not LXRβ. (A) Chemical structures of RC and RF. (B) CV-1 cells were transfected with a reporter plasmid (pLXRE$_X$4-tk-*Luc*) and expression plasmids for LXRα (or LXRβ) and RXRα, together with a β-galactosidase as an internal control, and were treated for 24 h with various concentrations of RC, RF, or 22(*R*)HC. Luciferase activity in the cell extracts was normalized using β-galactosidase and expressed as the fold induction relative to vehicle-treated cells. The values are the means ± S.D. of six experiments. (C) Effects of RC and RF on transcriptional activities of heterodimers between RXRα and RARα, RARβ, RARγ, FXR, LXRα, or LXRβ, and RXRα homodimers. CV-1 cells were transfected with expression plasmids for receptors and a β-galactosidase together with a reporter plasmid (pDR5$_X$3-cmv-*Luc* for RARs, pFXRE$_X$4-tk-*Luc*, or pDR1$_X$4-cmv-*Luc* for RXRα), or a PPARγ-Gal4-expression plasmid with a pGal4-UAS-*Luc*. The cells were exposed to RC (30 μM), RF (30 μM), or receptor-specific agonists (3 μM all-trans retinoic acid for RARs and RXRα, 30 μM chenodeoxycholic acid for FXR, 20 μM 22(*R*)HC for LXRs, 30 μM ciglitazone for PPARγ) for 24 h before assaying luciferase activity. The values are the means ± S.D. ($n = 3$). (D) Purified GST-LXRα or GST-LXRβ ligand binding domain was incubated with a fluorescence-tagged SRC-1 peptide and various concentrations of RC, RF, or 22(*R*)HC. The association of ligand-induced SRC-1 peptide with the receptor was monitored by evaluating the increases in millipolarization fluorescence units (mP). The values are the means ± S.D. of six experiments.

2.3. Coactivator association assay using fluorescence polarization

A coactivator association assay using fluorescence polarization was performed according to a previously described method [16]. A TAMRA-labeled peptide (0.1 μM with the amino acid sequence ILRKLLQE) was incubated with purified 1.5 μM GST-fused human LXRα ligand binding domain or LXRβ ligand binding domain in 100 μl of buffer (10 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, and 5 mM DTT at pH 7.9) in 96-well black polypropylene plates. After 1-h incubation at room temperature, ligand-dependent recruitment of the coactivator-peptide was measured as the increase in fluorescence polarization using a Fluorescence Plate Reader Fusionα (Perkin-Elmer Life Science).

2.4. Real-time quantitative reverse transcription-PCR

Gene-specific mRNA quantitation was performed by real-time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems). THP-1 cells maintained in RPMI 1640 medium (Sigma) containing 10% FCS were subcultured in 6-well plates and treated with test compounds in RPMI 1640 containing 0.2% bovine serum albumin. Total RNA extracted from cells using an RNeasy Kit (Qiagen) was treated with DNase according to the manufacturer's instructions. The relative mRNA expression levels were determined using the TaqMan one-step RT-PCR Master Mix (PE Applied Biosystems). The primer/probe sequences for human ABCA1, ABCG1, and SREBP-1c have been previously reported [17].

2.5. Measurement of lipid efflux to apolipoprotein A-I

The lipid efflux measurements were performed according to a previously described method [18]. Briefly, THP-1 cells were treated with the test compounds and 0 or 10 μg/ml of human apoA-I, isolated from the plasma HDL fraction, in RPMI 1640 containing 0.2% bovine serum albumin for 24 h. The lipids were extracted and the cholesterol level was determined using enzymatic methods.

2.6. Statistical analysis

Data were analyzed by ANOVA followed by the Student–Newman–Keuls method.

3. Results

3.1. Identification of RC as an LXRα Agonist

To discover novel LXR agonists, we screened a variety of natural products using a transient transfection assay. CV-1 cells were cotransfected with an LXRE-driven luciferase reporter plasmid and expression plasmids for human LXRα (or LXRβ) and RXRα. RC and RF (Fig. 1A) are novel macrocyclic bis(bibenzyl) dimers isolated from the liverwort [14,15]. Upon cotransfection with LXRα/RXR, RC (30 μM) raised the transactivation of the reporter gene by approximately 15-fold, while a natural LXR agonist, 22(*R*)-hydroxycholesterol (22(*R*)HC), resulted in a 30-fold increase (Fig. 1B). At higher concentrations, RC caused a decrease in β-galactosidase activity. LXRβ/RXRα-dependent transactivation was unaffected by this compound. RF, a 14-methoxyl derivative of RC, activated neither LXRα nor LXRβ. Furthermore, neither RC nor RF activated heterodimers between RXRα and RARα, RARβ, RARγ, FXR, or PPARγ, and RXRα homodimers in cell-based luciferase assays (Fig. 1C).

In an in vitro coactivator-recruitment assay, RC (up to 10 μM) induced a dose-dependent interaction between SRC-1

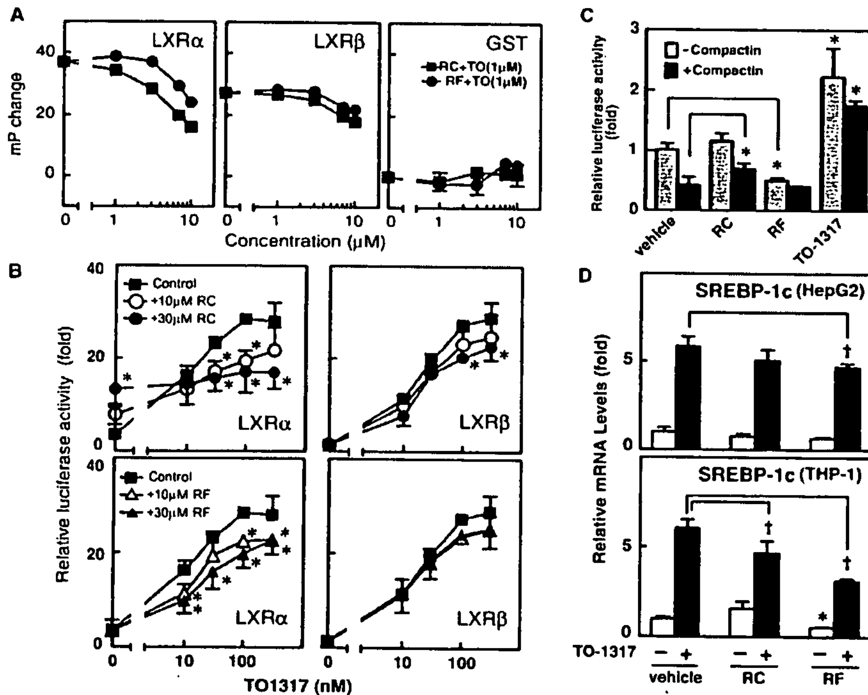

Fig. 2. RC competes with endogenous or synthetic ligands for LXR activation. (A) Fluorescence-tagged SRC-1 peptide was incubated with GST-LXRα, GST-LXRβ, or GST. The changes in fluorescence polarization caused by 1 μM TO-1317 in the presence of the indicated concentrations of RC or RF are shown. A blank value in the absence of ligands was subtracted from the measured values. The values are the means ± S.D. ($n = 3$). (B) CV-1 cells were transfected with pLXRE$_x$4-tk-*Luc* and either LXRα/RXRα or LXRβ/RXRα plasmids, as shown in Fig. 1B, and treated for 24 h with various concentrations of TO-1317 with or without RC or RF (10 or 30 μM). The values are the means ± S.D. of three experiments performed in triplicate. Data were normalized to no ligand control (take as 1) and 100 nM TO-1317 values of a representative experiment. Significantly different from respective controls (*, $P < 0.05$). (C) HepG2 cells were transfected with pLXRE-tk-*Luc* and treated for 24 h with RC (30 μM) or TO-1317 (10 nM) with or without compactin (50 μM) and mevalonic acid (40 μM). The values are the means ± S.D. of three experiments performed in triplicate. Significantly different from respective controls (*, $P < 0.05$). (D) THP-1 cells and HepG2 cells were treated for 24 h with the vehicle (DMSO) alone, RC or RF (30 μM) in the presence or absence of 100 nM TO-1317. SREBP-1c mRNA level was measured by TaqMan quantitative real-time PCR. Data were normalized to 18S rRNA levels and are expressed as the fold induction relative to that in the vehicle-treated cells. The values are the means ± S.D. ($n = 3$). Significantly different from vehicle controls (*, $P < 0.05$) or cells treated with TO-1317 alone (†, $P < 0.05$).

peptide and LXRα but not LXRβ (Fig. 1D). At higher concentrations, RC caused non-specific interference in monitoring fluorescence polarization. No interactions were induced by RF.

3.2. RC and RF compete with synthetic or endogenous ligands for LXR activation

When assayed in the presence of 1 μM TO-1317 (TO-901317), RC and RF reduced the TO-1317-induced association of the SRC-1 peptide with LXRα or LXRβ, indicating the binding of these compounds to the receptors (Fig. 2A). These compounds also decreased LXRα-transactivation elicited by a synthetic LXR agonist TO-1317 (Fig. 2B), although inhibitory effect of RF on LXRβ was insignificant. The ability of RC and RF to compete with endogenous ligands was tested in HepG2 cells. Treatment of the cells with compactin, which has been reported to deplete endogenous ligands for LXR [19,20], led to a 60% reduction in LXRE-dependent transactivation (Fig. 2C). Similarly, RF decreased the luciferase activity to the level of cells treated with compactin. RC had no effect in the absence of compactin but increased luciferase activity by 1.6-fold in compactin-treated cells. RC and RF inhibited the TO-1317-elicited expression of SREBP-1c mRNA in THP-1 cells. RF also decreased the TO-1317-elicited expression in HepG2 cells, although the effect of RC was insignificant (Fig. 2D).

3.3. RC enhances ApoA-I-mediated cellular cholesterol release

We used real-time quantitative PCR to investigate the effect of RC on the expression of LXR target genes, ABCA1, ABCG1, and SREBP-1c, in THP-1 cells. RC (30 μM) increased the ABCA1 mRNA level by 2-fold, while 22RHC (12.5 μM) and TO-1317 (10 nM) caused 2.2- and 2.7-fold inductions, respectively (Fig. 3A). Likewise, RC raised the ABCG1 and SREBP-1c mRNA level by 2.6-fold and 1.6-fold, respectively.

To evaluate whether the increase in ABCA1 mRNA expression was functionally relevant, we examined the effect of RC on cholesterol efflux from THP-1 cells. As shown in Fig. 3B, apoA-I-dependent cholesterol release was increased 2-fold by 10 μM of RC. At 30 μM, this compound also caused a 2-fold elevation in cholesterol release without exogenous apoA-I.

RC had no effect on SREBP-1c and ABCG1 mRNA expression in HepG2 cells, whereas TO-1317 markedly induced expression of both of them (Fig. 3C).

4. Discussion

In the present study, we identified RC as an LXRα-selective agonist. RC bound directly to LXRα and recruited the coacti-

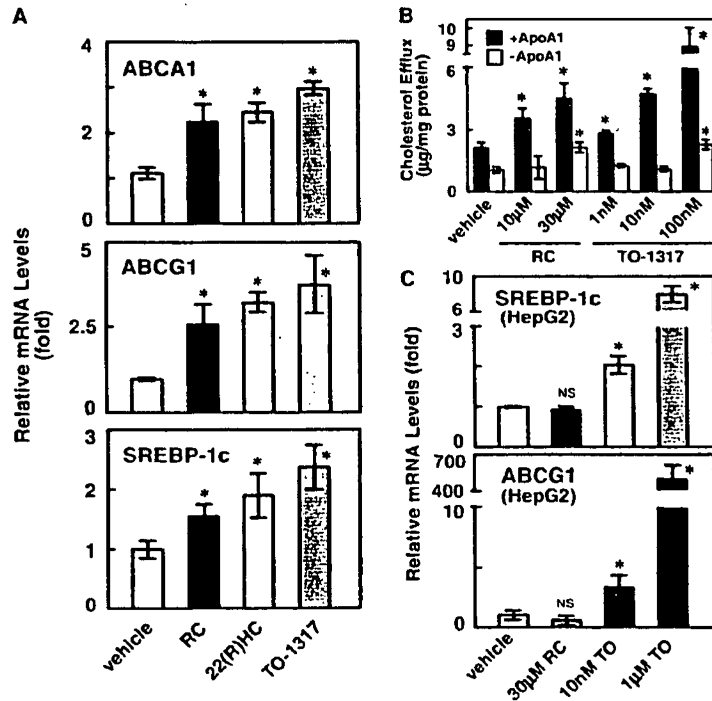

Fig. 3. RC increases ABCA1 and ABCG1 mRNA expression and cellular cholesterol efflux in THP-1 cells without raising SREBP-1c expression in HepG2 cells. (A) THP-1 cells were treated for 24 h with the vehicle (DMSO) alone, RC (30 μM), TO-1317 (10 nM), or 22(*R*)HC (12.5 μM). The levels of ABCA1 and ABCG1 mRNA were measured with TaqMan quantitative real-time PCR analysis. Data were normalized using the 18S rRNA levels and are expressed as the fold induction relative to that in the vehicle-treated cells. The values are the means ± S.D. of three experiments performed in triplicate. (B) Cells were treated for 24 h with the indicated compounds in the presence or absence of apoA-I (10 μg/ml), and the cholesterol released into the medium was analyzed. The values are the means ± S.D. (n = 3) of a typical series of three experiments performed. (C) HepG2 cells were treated for 24 h with the vehicle (DMSO) alone, RC (30 μM), or TO-1317 (10 nM or 1 μM). SREBP-1c and ABCG1 mRNA level was measured by quantitative real-time PCR. Data were normalized to 18S rRNA levels and are expressed as the fold induction relative to that in the vehicle-treated cells. The values are the means ± S.D. of three experiments. Statistically significant differences from control are indicated by asterisk (*, $P < 0.05$ and NS, not significant).

vator SRC-1 peptide to the receptor (Fig. 1D), leading to the activation of LXRα-dependent reporter gene transcription (Fig. 1B). RC also possesses the ability to bind to LXRβ. However, its inability to recruit a coactivator to LXRβ (Fig. 1D) resulted in the failure of LXRβ-mediated transactivation. RC was shown to have no ability to activate PPARγ, RARα, RARβ, RARγ, FXR, and RXRα (Fig. 1C).

In contrast to RC, RF was inactive for both LXRα and LXRβ-mediated transactivation (Fig. 1B). This compound was able to bind to LXRα and LXRβ (Fig. 2A) but was unable to promote coactivator association (Fig. 1C). Structural differences between RC and RF suggest that the C-14 hydroxyl group of RC plays a critical role in inducing coactivator association to LXRα, but not to LXRβ, leading to transactivation in a subtype-selective manner.

The competition with the synthetic agonist TO-1317 for the coactivator association (Fig. 2A) and the receptor-mediated transactivation (Fig. 2B) demonstrates that RC functions as an LXRα partial agonist and an LXRβ antagonist. RF functions as an antagonist of LXRα. RF decreased LXRE-dependent luciferase transcription in HepG2 cells to the level of endogenous-ligand depletion by compactin (Fig. 2C), suggesting that endogenous ligand-mediated LXR activation was inhibited by RF. RC increased LXRE-dependent transcription in compactin-treated cells, but not in the absence of compactin. In the intact cells, RC, as well as RF, might compete with endogenous ligands for LXR activation, thereby decreasing endogenous ligand-mediated activation. This latent activity of RC might compensate for this reduction. RC and RF also competed with TO-1317 for SREBP-1c expression in THP-1 cells.

The efflux of cellular cholesterol to HDL constitutes the first stage in the reverse cholesterol transport pathway and plays a critical role in modulating the progression of atherosclerosis [21]. ABCA1 and ABCG1 have been shown to mediate this process [22,23], and LXRα and LXRβ play redundant roles in their expression [9,24]. RC functions as an LXRα partial agonist and LXRβ antagonist. However, RC was shown to raise ABCA1 and ABCG1 mRNA levels and enhance cellular cholesterol efflux in THP-1 cells (Fig. 3A and B). Because human LXRα expression is autoregulated by LXRα itself [25], the stimulation of LXRα may have a stronger effect than that of LXRβ. RC and TO-1317 at higher concentrations increased cholesterol efflux even in the absence of apoA-I. Because THP-1 cells express apoE [26] and ABCA1 at the basal level, increased expression of apoE by LXR activation [27], caveolin-1 [28], or ABCA7 [29] could lead to enhanced cholesterol release, even without apoA-I.

We found that RC did not affect SREBP-1c and ABCG1 expression in hepatoma HepG2 cells (Fig. 3C). This coincides with the apparent inability of RC to enhance LXRE-dependent transactivation in this cell line (Fig. 2C). A possible explanation for this inability is the low LXRα/LXRβ ratio in HepG2 cells. Alternatively, as a partial LXRα agonist/LXRβ antagonist, RC might compete with endogenous ligands produced via the cholesterol synthesis pathway. It is also possible that coactivator-corepressor(s) different from those employed in THP-1 cells might be active in this cell line. Further studies are required to elucidate the precise mechanisms of RC's selective actions. However, because synthetic LXR agonists have been shown to cause hypertriglyceridemia [5], LXR modulators that do not upregulate hepatic lipogenic genes may enable improved therapeutic strategies.

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Thyroid Hormone Targets Matrix Gla Protein Gene Associated With Vascular Smooth Muscle Calcification

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Abstract—Thyroid hormones have marked cardiovascular effects in vivo. However, their direct effects on vascular smooth muscle cells have been unclear. Because thyroid hormones play critical roles in bone remodeling, we hypothesized that they are also associated with vascular smooth muscle calcification, one of the pathological features of vascular sclerosis. To test this hypothesis, we examined the effects of 3',3,5-triiodo-L-thyronine (T_3) on the expression of calcification-associated genes in rat aortic smooth muscle cells (RAOSMCs). Quantitative RT-PCRs revealed that a physiological concentration of T_3 (15 pmol/L free T_3) increased mRNA level of matrix Gla protein (MGP), which acts as a potent inhibitor of vascular calcification in vivo, by 3-fold in RAOSMCs, as well as in cultured human coronary artery smooth muscle cells. In RAOSMCs transiently transfected with a luciferase reporter gene driven by the MGP promoter, T_3 significantly stimulated luciferase activity. In addition, RNA interference against thyroid hormone receptor-α gene diminished the effect of T_3 on MGP expression. Aortic smooth muscle tissues from methimazole-induced hypothyroid rats (400 mg/L drinking water; 4 weeks) also showed a 68% decrease in the MGP mRNA level, as well as a 33% increase in calcium content compared with that from the control euthyroid animals, whereas hyperthyroidism (0.2 mg T_3/kg IP; 10 days) upregulated MGP mRNA by 4.5-fold and reduced calcium content by 11%. Our findings suggest that a physiological concentration of thyroid hormone directly facilitates MGP gene expression in smooth muscle cells via thyroid hormone nuclear receptors, leading to prevention of vascular calcification in vivo. (*Circ Res*. **2005;97:550-557.**)

Key Words: calcium ■ gene expression ■ nuclear receptors ■ vascular smooth muscle ■ thyroid hormone

Thyroid hormone has marked effects on differentiation, development, and metabolic balance of virtually every body tissue. The action of thyroid hormone is mediated by high-affinity thyroid hormone nuclear receptors (TRs), which recognize specific response elements in the promoters of target genes and regulate their transcriptional activity in response to the hormone. Alterations in thyroid hormone levels have a profound impact on the cardiovascular system, which include changes in myocardial contractility, heart rate, and resistance of peripheral vasculature. Hyperthyroidism leads to positive inotropic, lusitropic, and chronotropic effects on the heart and low systemic vascular resistance, whereas the opposite is observed in hypothyroidism. In myocardium, the mechanisms for these changes are based on altered expression levels of several key proteins involved in the regulation of intracellular ion homeostasis. The effects of thyroid hormone on cardiac contractility as well as rates of contraction and relaxation are mainly mediated by increases in the levels of the sarcoplasmic reticulum Ca^{2+}-ATPase and decreases in its inhibitor phospholamban in cardiomyocytes.[1] The positive chronotropic effect of thyroid hormone is associated with altered expression levels in plasmalemmal ion channels/transporters in the heart, such as Kv1.5, Kv4.2, minK, hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), HCN4, Na^+-Ca^{2+} exchanger, and Na^+-K^+-ATPase.[2–5] In contrast, although ≈25% of hypothyroid patients have diastolic hypertension,[6] the mechanism for the altered systemic vascular resistance under an abnormal thyroid hormone status is not well understood. To date, a loss of nongenomic vasodilating action of thyroid hormone[7] and atherosclerosis attributable to hypercholesterolemia[8] have been associated with the increased systemic vascular resistance under hypothyroidism.[9] Recently, mRNAs for TR isoforms were identified in aortic and coronary smooth muscle cells, suggesting that a direct genomic action of thyroid hormone may play a significant role in vascular smooth muscle.[10] Although extremely high concentrations of thyroid hormone are known to regulate expression of several genes in vascular smooth muscle cells,[10,11] the physiological and direct target genes of thyroid hormone in vascular smooth muscle cells are not known.[9]

Arterial calcification is a common pathological feature of vascular sclerosis, as well as a variety of metabolic disorders such as diabetes and renal disease. Decades ago, cretinism

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