

tween the N-CoR C terminus and AR was ligand independent. Moreover, in the mammalian two-hybrid assay for AR–N-CoR interaction analysis, the authors used AR–ligand binding domain, not the full-length of the receptor. In fact, in a recent study by Hodgson et al., DHT-dependent interaction between the C terminus of N-CoR and the full-length AR was not detected (20), which corresponds with our present results (Fig. 1 and 6). Therefore, it is supposed that the middle region may be important for the agonist-dependent interaction between N-CoR and AR, whereas the C terminus of N-CoR may be important for the antagonist-induced interaction. In the present study, the middle region of the N-CoR, N-CoR(1134–1798), but not the amino- or carboxyl-terminal region of the N-CoR, was recruited by agonist-bound AR, GR α , and ER α in living cells (Fig. 5). Furthermore, coimmunoprecipitation experiments revealed that N-CoR(1134–1798) containing RD3 was able to interact with the AR. Therefore, N-CoR(1134–1798) containing RD3 is strongly suggested to be the major region for the repression of transactivation function of agonist-bound steroid hormone receptors. The N-CoR(1–1133) also contributes to the interaction with agonist-bound receptors but does not seem to be responsible for the repression of their transactivation function (Fig. 6 and 7).

Many coactivators have been shown to regulate AR transactivation function through an interaction with the N-terminal domain of the AR. The p160 coactivators such as SRC-1 and TIF2 directly interacted with the N-terminal domain of the AR (2). It has previously been reported that SRC-1 and TIF2 were recruited into the AR–AF-1 foci (41). The N-CoR protein was also shown to interact with the N-terminal domain of steroid hormone receptors (20, 44, 60). In the present study, the N-CoR was also recruited to the AR–AF-1 foci, and the interaction between N-CoR and AR–AF-1 was confirmed by mammalian one-hybrid assay. The N-CoR is thus suggested to repress AR-mediated transactivation through an interaction with the N-terminal domain of the AR.

We demonstrated that the N-CoR inhibited the AR N-C interaction in the mammalian two-hybrid assay and also in the AR-mediated transactivation. A specific HDAC inhibitor, TSA, could not recover the N-CoR-mediated suppression of AR-dependent transactivation (Fig. 12A). It has been recently reported that SMRT-mediated inhibition of the steroid hormone receptor function is not recovered by TSA (1, 23) and is largely due to the blockage of the AR N-C interaction (1, 34). In the present study, we confirmed that N-CoR also had such inhibitory effects on the AR N-C interaction. In a recent paper (32), it has been reported that the intramolecular N-C interaction controls AR chromatin binding and is required for the recruitment of SWI/SNF, which remodels chromatin. Inhibition of intramolecular interactions by N-CoR might affect chromatin remodeling, resulting in repression of the transactivation function.

Recently, it was proposed that coactivators and corepressors exert equilibrium interactions with nuclear receptors (61) and that the properties of nuclear receptors are determined by the intracellular ratio of coactivators and corepressors (45, 51, 61). It was also proposed that the transcriptional regulation by nuclear receptors requires the corepressor-coactivator exchange via the TBL1/TBLR1-containing exchange complex (39). Competition between N-CoR and coactivators to regu-

late N-C interaction and the transactivation activity of AR was revealed in the present study, which seems consistent with the above hypothesis.

The appearance of intranuclear focus formation has been shown to be closely related to the transcriptionally active conformation of steroid hormone receptors with coactivators (13, 30, 41, 56). Upon treatment with DHT, AR-GFP alone formed complete foci with recruitment of coactivators such as SRC-1, TIF2, and CBP (41). Such focus formation was clearly demonstrated for endogenous AR in the present study (Fig. 8A). Treatment with some antagonists, which inhibit the transcriptional activation, translocates the receptors from the cytoplasm into the nucleus; however, the translocated receptors are not able to form intranuclear foci and are diffusely distributed (13, 56). The N-C interaction is also required for complete focus formation of ARs (41). However, the present study clearly demonstrated that the quality of AR foci or ER α foci containing the N-CoR was different from that of AR foci or ER α foci without N-CoRs. The focus pattern of AR-GFP and ER α -GFP without N-CoRs (Fig. 2Ab and Gb and 9A and K) seemed much more distinct than the speckle pattern of AR-GFP and ER α -GFP coexpressed with the N-CoR (Fig. 2Ci and Ii and 9C and M), but such differences in the appearance of the intranuclear compartments were not confirmed only by simple two-dimensional images. However, a quantitative analysis of fluorescence intensity fluctuation in two-dimensional images and a three-dimensional imaging method we previously developed (41, 56) clearly demonstrated impaired formation of AR or ER α foci (incomplete focus formation) in the presence of the N-CoR (Fig. 9 and 10). The transactivation activities of AR and ER α were parallel at the level of their distinct (complete) focus formation (Fig. 6 and 10). It was thus suggested that only complete focus formation is related to the full transactivation function of ARs. An antagonist, tamoxifen, was shown to induce ER α focus formation, which was similar to foci induced by estradiol (Fig. 8Bd and Ca). However, the present study demonstrated that the tamoxifen-induced foci of ER α are not complete foci (Fig. 8Cb and Cc). The biochemical analyses using the two-hybrid and reporter gene assays revealed the functional competition between the N-CoR and CBP/SRC-1 on transactivation activity of the AR. This finding would further conceive that the N-CoR and coactivators mutually and exclusively bind to ARs, namely, binding of CBP and SRC-1 leads to a change in molecular conformation of the AR that hinders further binding of N-CoRs and vice versa. The present three-dimensional imaging analysis provided evidence for the above concept. Additional expression of SRC-1 or CBP in DHT-bound ARs coexpressed with N-CoRs led to release of N-CoRs from incomplete foci containing DHT-bound ARs and N-CoRs and to formation of distinct/complete foci with the coactivators (Fig. 13). Essentially the same phenomena were observed for endogenous N-CoR (Fig. 14). We speculate that there is a conformational equilibrium between transcriptionally active and inactive steroid hormone receptors in the presence of agonists, determined by the relative ratio of coactivators and corepressors.

The present observations that transcription inhibitors, actinomycin D and α -amanitin, disrupt intranuclear foci of steroid hormone receptors (Fig. 8B) revealed that the focus formation is considered not to precede the transcriptional activation. The

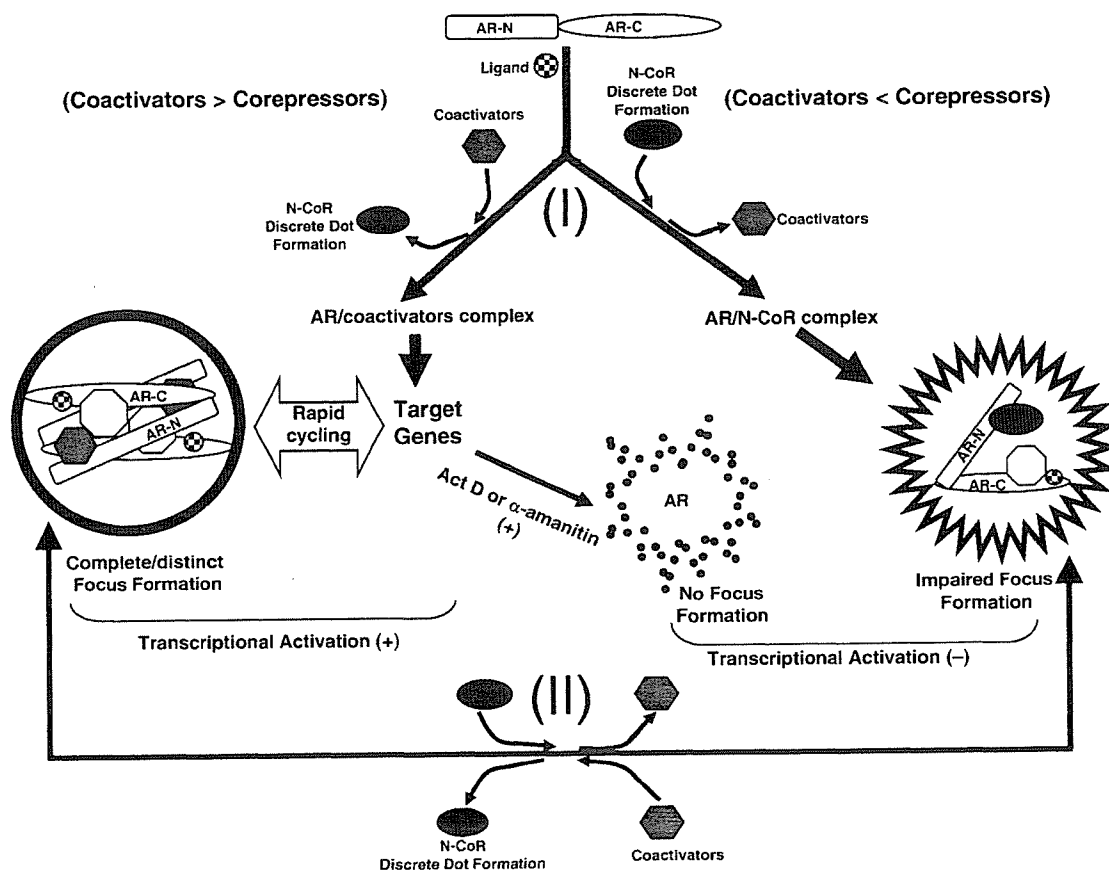


FIG. 15. Speculation of N-CoR action on intranuclear focus formation and transactivation function of the AR. DHT induces a conformational change of the AR and binding domains for coactivators, and the corepressors, N-CoRs, in the amino termini of the AR are unmasked. Under the intranuclear environmental conditions in which the content of the N-CoR is considerably low compared with that of coactivators, DHT-bound AR takes proper N-C interaction with bound coactivators and forms a transcriptionally active conformation, and the AR-coactivator complexes transactivate on the target genes. The transcriptionally active AR-coactivator complexes undergo a rapid cyclic exchange between the subnuclear compartment (complete/distinct foci) and transcriptionally active sites of the target genes. When transcription is prevented by a transcription inhibitor, actinomycin D or α -amanitin, a rapid exchange may be inhibited and then the focus formation is destroyed. The appearance of complete/distinct foci would be an indicator for the transcription active status. Under the environmental conditions in which the N-CoR content is relatively high, the N-CoR binds to liganded AR (apparent overlapping) and interferes with the N-C interaction, resulting in a transcriptionally inactive conformation, which shows the intranuclear impaired focus formation. There are several possible phases (I and II) for the conformational equilibrium between transcriptionally active and inactive DHT-bound AR dependent on the relative ratio of coactivators and N-CoR.

steroid hormone receptors and coactivators show multiphasic on and off switching for binding to promoter elements of the target genes. The receptor-coactivator complexes also undergo a rapid exchange between the target genes and their subnuclear compartments (34, 35, 48). Based on these reported observations and the present findings that complete focus formation is related to the transcriptional activation, it is suggested that complete focus formation of steroid hormone receptors reflects the accumulation of steroid hormone receptor-coactivator complexes released from their target genes. Therefore, it is further suggested that focus formation is a mirror of transcriptionally active complex formation of steroid hormone receptors and coactivators at transcription sites. Thus, investigation of focus formation would be useful for the elucidation of the mechanism of steroid hormone receptor-dependent transactivation in living cells. A physiological role for focus formation still remains to be clarified. One speculated possibility is as follows. Numerous important molecules are functioning in the nucleus. Some regulatory mechanisms for

these molecules should be present. Focus formation might be one of these mechanisms, and foci function as temporary storing and buffering sites that are related to steroid hormone receptor cycling. Figure 15 summarizes the speculation of the intranuclear compartmentalization and transactivation function of the AR based on the present findings.

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Pivotal role of Notch signaling in regulation of erythroid maturation and proliferation

Tachikawa Y, Matsushima T, Abe Y, Sakano S, Yamamoto M, Nishimura J, Nawata H, Takayanagi R, Muta K. Pivotal role of Notch signaling in regulation of erythroid maturation and proliferation.

Abstract: Notch signaling plays an important role in cell fate decisions in developmental systems. To clarify its role in committed hematopoietic progenitor cells, we investigated the effects of Notch signaling in erythroid colony forming cells (ECFCs) generated from peripheral blood. ECFCs express Notch receptors, Notch1 and Notch2, and Notch ligands Delta1, Delta4, and Jagged1. When we assayed the effects of Notch ligands on erythroid maturation by flow cytometry, we found that immobilized Delta1 and immobilized Delta4 in particular inhibited maturation, whereas Jagged1 had no effect. In addition, Delta4 inhibited proliferation without reducing cell viability. Increases in expression levels of the Notch target gene hairy enhancer of split (HES) -1 were evident by real-time PCR after stimulation with immobilized Delta4. The effect of soluble Delta4 on expression of HES-1 was less pronounced than that seen with the immobilized form, indicating that all surface-bound ligands are important for effective signal transduction. When ECFCs were cultured in the presence of soluble Delta4 at a low cell concentration, erythroid maturation was slightly inhibited, but at a high concentration, maturation was promoted via competition of soluble Delta4 with endogenous ligands. These results indicate a pivotal role of Notch signaling in regulating erythroid maturation and proliferation, and further suggest that cell-cell interactions modulate growth of erythroid progenitor cells via Notch system.

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Key words: notch signalling; ECFCs; notch ligand; delta4; erythroid progenitor cells; normal hematopoietic cells

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Notch receptors play an important role in cell fate decisions by binding to their ligands on adjacent cells in various developmental systems (1–3). At present, four Notch receptors, Notch-1, -2, -3, -4 (4–6), and their ligands, Delta-1, -3, and -4, and Jagged-1 and -2 (7–10), have been identified in mammals. Notch ligands bind to Notch receptors through their extracellular domains, trigger proteolytic processing, and release the Notch intracellular domain (NIC) from the cell membrane. Cleaved NIC interacts with the DNA-binding transcription factor CBF1/RBP-J κ , and the complex translocates into the nucleus (11). This complex binds to the Hairy enhancer of split (HES) -1 promoter and stimulates the transcription of the HES-1 gene (1, 12). Moreover, over-expressed NIC acts as a constitutively active form of Notch receptor (13, 14).

Recently it has been demonstrated that cell-cell signaling via Notch regulates lineage commitment

at various points during hematopoiesis. For example, NIC transgenic mice have defective B-cell development, and Notch signaling influences the decision to differentiate into $\alpha\beta$ or $\gamma\delta$ T cells (15), as well as that of differentiation into CD4 or CD8 T cells (16). These observations indicate that Notch signaling plays a central role in regulation of cell fate decisions of multi- or bi-potent precursors (17, 18). Notch receptors expressed by bi-potential progenitors are activated by Notch ligands expressed on neighboring progenitors, leading to inhibition of differentiation of the Notch-expressing cells along a fate-specific pathway. These cells remain undifferentiated or differentiate along an alternate pathway in the presence of appropriate stimuli (namely, lateral inhibition) (1). The Notch system is known to play crucial roles in the regulation of blood cell production, modulation of proliferation, and lineage commitment. Even so,

the role of Notch signal in growth of committed progenitors is not fully understood.

Human erythroid progenitor cells purified from peripheral blood (erythroid colony forming cells; ECFCs) are committed erythroid progenitor cells and it has been known that in erythroid maturation and proliferation, several growth factors, such as interleukin-3 (IL-3), erythropoietin (EPO), stem cell factor (SCF), and insulin-like growth factor 1 (IGF 1), play essential roles at each maturation stage. Highly purified peripheral erythroid burst-forming units (BFU-E) are dependent on IL-3 for the first 2–3 d of *in vitro* culture. SCF is required in the earliest BFU-E through day 8 ECFCs. ECFCs proliferate and mature from erythroid progenitor cells into late erythroblasts in serum-free cultures with EPO and IGF-1 (19). However, the role of Notch signaling in these committed human erythroid progenitor cells has not yet been investigated.

In this study, we investigate whether components of the Notch pathway are expressed in human ECFCs and whether Notch signaling influences erythroid maturation. We found that components of the Notch pathway were expressed in human erythroid progenitor cells and that maturation and proliferation of these cells can be altered by activation of the Notch signaling pathway. The results suggest that cell–cell interactions modulate growth of erythroid progenitor cells via the Notch system.

Materials and methods

Reagents

Recombinant human erythropoietin (rhEPO) was kindly provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan); recombinant human interleukin 3 (rhIL-3), and recombinant human stem cell factor (rhSCF) were kindly provided by Kirin-Brewery Co Ltd (Tokyo, Japan). Mouse anti-IgG1 antibody was purchased from Life Technologies (Rockville, MD, USA), and human IgG1 kappa from The Binding Site (Birmingham, UK).

Purification and expansion of ECFCs

The ECFCs were prepared as previously described (20–22). Briefly, light-density mononuclear cells were obtained from 40 mL of heparinized peripheral blood buffy coat from healthy Japanese volunteers after informed consent. Cells were isolated by density centrifugation using lymphocyte separation medium (LSM, density 1.0770–1.0800 g/mL; ICN Biomedicals, Aurora, OH, USA). Red blood cells were lysed by suspending the mononuclear cell pellet in red cell lysis buffer (0.16 mol/L

ammonium chloride, 10 mmol/L potassium bicarbonate, 5 mmol/L EDTA). Platelets were removed by cell centrifugation through phosphate-buffered saline (PBS) containing 10% human serum albumin (HSA, kindly provided by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). Adherent cells were depleted by a 1-h incubation in a polystyrene tissue culture flask at 4 °C. Non-adherent cells were then collected and two cycles of negative selection were performed using anti-CD3, -CD11b, -CD15, and -CD45RA antibodies and immunomagnetic beads with VarioMACS columns (Miltenyi Biotech, Auburn, CA, USA). The remaining cells were cultured in Iscoves modified Dulbecco medium (IMDM; GIBCO BRL, Grand Island, NY, USA) containing 15% heat-inactivated fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Vic., Australia), 15% pooled human AB serum, 2 U/mL EPO, 20 ng/mL SCF, 10 ng/mL IL-3, 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO) at 37 °C in a high humidity, 5% CO₂, 95% air incubator (day 0). On day 3, the cells, referred to as day 3 ECFCs, were centrifuged over LSM, then collected and incubated under the same conditions, but without IL-3. The purity of day 3 ECFCs, with proerythroblastic features, was 79.8% ± 11.6%, as determined by cytospin preparation. Cell purity was assessed in each experiment.

Serum-free liquid culture of day 3 ECFCs

Day 3 ECFCs (1.0×10^4 cells/mL) were incubated in serum-free liquid medium containing 50% IMDM/50% F-12 medium (Sigma Chemical, St Louis, MO, USA) with 0.1% detoxified bovine serum albumin (BSA; Stem Cell Technologies, Vancouver, BC, USA), 300 µg/mL iron-saturated transferrin (652202, Boehringer Mannheim, Mannheim, Germany), lipid suspension prepared, as described (21), 10 U/mL rhEPO, and 50 ng/mL rh SCF at 37 °C in a high humidity, 5% CO₂, 95% air incubator. Cultured cells were collected on days 5, 7, and 9 (referred to as day 5 ECFCs, day 7 ECFCs, and day 9 ECFCs).

Cloning and expression of human Notch receptors and ligands

Purified human Notch ligand-immunoglobulin (Ig) G1 chimeric proteins and a human Delta4-FLAG octapeptide chimeric protein were prepared as described previously (23). Briefly, isolation of a complementary DNA (cDNA) clone of human Notch ligands was performed by plaque hybridization. cDNA encoding the extracellular domain of Notch ligands was fused in frame to a sequence of human IgG1 or of FLAG octapeptide. The Notch

ligand-IgG or -FLAG fusion proteins were produced by transfection of the fused genes in the expression vector pcDNA3 or pM-KITneo into Chinese hamster ovary cells via electroporation, followed by G418 selection. The Notch ligand fusions were detected using anti-human IgG1 or -FLAG antibody. The chimeric proteins were purified from culture supernatants by affinity chromatography and stored in PBS. The purity was more than 90%, as determined by SDS-PAGE.

Determination of cell viability

Viability of the cells was determined by trypan blue exclusion using a hemocytometer.

MTT colorimetric assay

Cell growth was examined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasodium bromide (MTT) colorimetric assay (Chemicon International Inc., Osaka, Japan). After incubation, cells were washed with IMDM and plated into serum-free liquid medium in 96-well tissue culture plates. Then MTT solution (1 mg/mL) was added to each well, and the cells were further incubated for 4 h at 37 °C. The absorbance at the wavelength of 570 nm was measured by a micro-ELISA reader. The negative control well contained medium only. The effect of additives on cell survival was estimated as a percentage of the value of untreated control.

Assessment of erythroid maturation by flow cytometry

To determine the effect of Notch ligands on erythroid maturation, expression of the transferrin receptor (TfR) and glycophorin A (GPA) on ECFCs were analyzed by flow cytometry as described (24). The cells (1.0×10^5) were washed twice with PBS and resuspended in PBS with 0.05% BSA. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-GPA MoAb (11E4B-7-6, IM2212; Immunotech, Marseille, France) and phycoerythrin (PE)-conjugated anti-CD71 MoAb (YDJ1.2.2, IM2001; Immunotech) for 30 min on ice. Samples were analyzed using the Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA).

Plasma clot assay

The erythroid colony-forming capacity of ECFCs was determined by the plasma clot method (22). Day 3 ECFCs were incubated for 6 d in serum-free liquid medium containing 50% IMDM/50% F-12 medium with 0.1% BSA, 300 µg/mL iron-saturated

transferrin, lipid suspension, 10 U/mL rhEPO, and 50 ng/mL rhSCF at 37 °C in a high humidity, 5% CO₂, 95% air incubator. The cells (day 9 ECFCs) were then collected, and 600 cells with the media consisting of IMDM, 20% FCS, 1% BSA, 10 ng/ml rhSCF, 2 U/ml rhEPO, and 10% citrated human AB plasma were plated in triplicate 35-mm culture dishes and incubated for an additional 7 d. The clots were then fixed and stained with 3, 3'-dimethoxybenzidine. The number of hemoglobinized cells in randomly selected colonies was counted for each plate.

Reverse transcriptase PCR (RT-PCR) and real-time quantitative RT-PCR

Total RNA was isolated from 10^6 cells with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using 1 µg total RNA by the RT-PCR kit (Takara, Shiga, Japan) according to the protocol of the manufacturer. To determine the expression of Notch signaling components, RT-PCR reactions for Notch receptors and ligands were performed in a Perkin-Elmer 9700 cyclor (Foster City, CA, USA) for a total of 40 cycles. Reactions were optimized for each set of primers as shown in Table 1.

To serve as an internal control, β-actin was amplified from all cDNA templates generated. PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed.

Quantitative real-time PCR assay of transcripts were carried out using gene-specific double fluorescently labeled probes in LightCycler Software Version 3.5 (Roche, ID, USA). Target quantities were normalized to 18S rRNA and calibrated using day 6 values and defined as a value of 1.0. All quantities were expressed as n-fold relative to the calibrator (day 6).

Statistical analysis

The Student's *t*-test was used to determine significant differences between the groups and results are expressed as the mean ± SD.

Results

Notch receptors and ligands are expressed on ECFCs

To determine the expression pattern of Notch receptors and ligands in erythroid progenitor cells, we examined the expression of all of four Notch receptors and three of five ligands, Delta1, Delta4, and Jagged1, during liquid culture of ECFCs via RT-PCR. As shown in Fig. 1A, Notch1 and

Table 1. Specific primer pairs used for RNA analysis

Gene	Size (bp)	Annealing temperature (°C)	5' primer	3' primer
Notch1	547	58	CAGGTCAGTACTGTACCGAG	TGGCACTCTGGAAGCACTGC
Notch2	398	58	ACATCATCACAGACTTGGTC	CATTATTGACAGCAGCTGCC
Notch3	659	58	TGGATGAGTGTACAGCTGCAG	AGGTGCAGCTGAAGCCATTG
Notch4	622	61	ACATACCCCCAGATCCAGTG	AGAGGCAGGAGAAAGAGCCC
Delta1	469	58	TGCAGGAGTTCGTCAACAAG	TCCGTAGTAGTGTTCGTAC
Delta4 first	656	58	GTTTCATCAACGAGCGCGGGC	GGGATGCATTTCGTACACAG
Delta4 second	642	62	TCAACGAGCGCGCGTAGTG	TCGTTACACAGCCGGCCCTG
Jagged1	436	58	GATCCTGTCCATGCAGAACG	GGATCTGATACTCAAAGTGG
HES-1	374	59	GACAGCATCTGAGCACAGAAATG	GTCATGGCATTGATCTGGGTCAT
β -actin	266	57	TACCTCATGAAGATCCTCA	TTCGTGGATGCCACAGGAC
18S rRNA	269	66	ACTCAACACGGGAAACCTCA	GGACATCTAAGGGCATCACA

18S rRNA Probe: LightCycler – Red 640 – 5' – TCCGATAACGAACGAGACTCTGGCAT – 3'

5' –GTTGGTGGAGCGATTGTCTGGTTAA – 3' – FITC

HES, hairy enhancer of split.

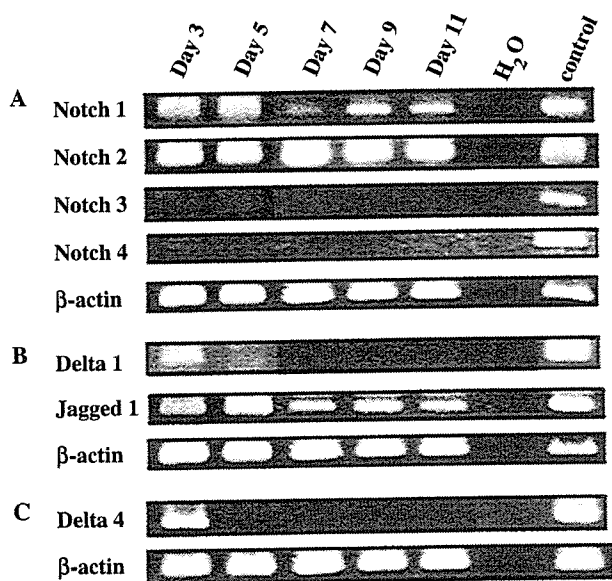


Fig. 1. Expression of Notch receptors and ligands on erythroid colony forming cells (ECFCs). Day 3 ECFCs (1.0×10^4 cells/well) were transferred to serum-free medium containing recombinant human erythropoietin (10 U/mL) and recombinant human stem cell factor (50 ng/mL). At the indicated times, RNA was extracted and subjected to RT-PCR followed by electrophoresis on an agarose gel and staining with ethidium bromide. (A) Expression of Notch-1, -2, -3, -4. Notch1 and Notch2 are expressed during erythroid maturation. Neither Notch3 nor Notch4 can be detected. (B) Expression of Delta1 and Jagged1. Delta1 is expressed only on day 3. Jagged1 is detected throughout erythroid maturation. (C) Expression of Delta4 as detected by nested PCR. The purity of ECFCs collected at day 3 ECFCs was $79.8\% \pm 11.6\%$; day 5, $91.8\% \pm 0.3\%$; day 7, $93.6\% \pm 5.1\%$; day 9, $98.5\% \pm 0.8\%$; and day 11, $99.8\% \pm 0.2\%$ (mean \pm SD).

Notch2 were expressed at all stages investigated. In contrast, Notch3 and Notch4 were not detected at any stage of maturation we tested. In addition, Delta1 and Delta4 were expressed only on day 3 ECFCs, and Jagged1 was expressed at all stages investigated (Fig. 1B and C). Although Delta4 was

not detectable in first-round RT-PCR, Delta4 transcripts could be detected by nested PCR in day 3 ECFCs. The purity of day 3 ECFCs prepared for RT-PCR was $79.8\% \pm 11.6\%$ after the second negative selection; the purity of day 5 ECFCs was $91.8\% \pm 0.3\%$; day 7 ECFCs, $93.6\% \pm 5.1\%$; day 9 ECFCs, $98.5\% \pm 0.8\%$; and day 11 ECFCs, $99.8\% \pm 0.2\%$. Regarding the stage of erythroid differentiation, days 7 and 9 ECFCs were in basophilic erythroblast stages and about 75% of day 11 ECFCs were in polychromatic stages and the others, orthochromatic.

Immobilized Delta1 and Delta4 inhibit maturation of ECFCs

The fact that ECFCs expressed both Notch receptors and their ligands suggested the possibility that Notch signaling induced by cell-cell interaction might affect the maturation of erythroid progenitors. Thus, we next examined the effects of Notch ligands on maturation of ECFCs. When day 3 ECFCs were incubated in serum-free media, the cells first began to express TfR and then these TfR+ cells expressed GPA; i.e., the percentage of mature cells (TfR+/GPA+) gradually increased during culturing (Fig. 2A, upper panel). When day 3 ECFCs were cultured with immobilized Delta4-IgG1 fusion proteins (30 mg/mL) that had been plated on 24-well plastic plates coated with BSA, the percentage of mature erythroid cells (TfR+/GPA+) was lower than the percentage detected in controls at any time point (Fig. 2A, lower panel).

Next, we looked at the effect of other Notch ligands, Delta1 and Jagged1, on maturation of ECFCs. On day 9, the percentage of TfR+/GPA+ cells was significantly lower after incubation with immobilized Delta1 and Delta4 (vs. control; Delta1, $P < 0.05$, Delta4, $P < 0.001$), whereas immobilized Jagged1 had no effect on erythroid maturation even at day 9 (Fig. 2B).

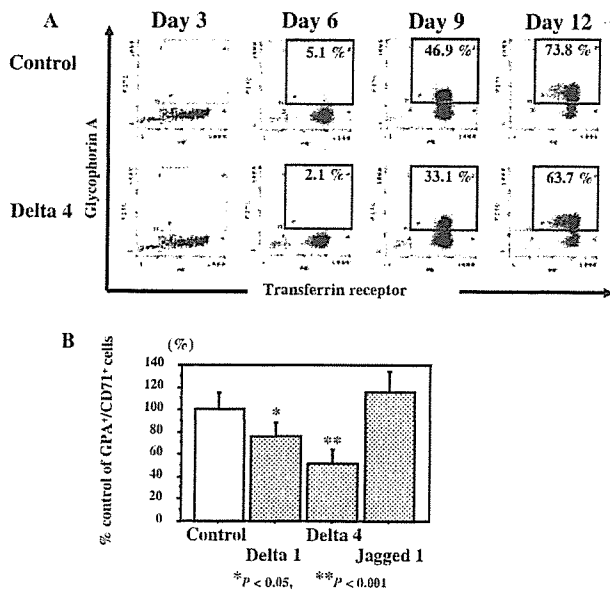


Fig. 2. Effects of immobilized Notch ligands on maturation of erythroid colony forming cells (ECFCs). Day 3 ECFCs (1.0×10^4 /mL) were cultured with human IgG1-kappa or immobilized Notch ligands (30 mg/mL) in serum-free medium and expression of transferrin receptor (TfR) and glycophorin A (GPA) was determined by flow cytometry at the indicated days. Data are representative of three experiments. Numbers in corners show the percentage of gated events. (A) Serial change of expression of GPA and TfR during erythroid maturation in control (upper panel) and with Delta4 (lower panel). (B) Each bar represents the ratio of mature cells (TfR +/GPA +) on day 9 after culture with Notch ligand to the number of mature cells in the control. Each point shows the mean \pm SD of triplicate experiments.

Immobilized Delta4 inhibits proliferation of ECFCs without reducing cell viability

Next we examined the effect of Notch ligands on cellular proliferation of ECFCs. Immobilized Delta4 significantly reduced the number of ECFCs without having an effect on viability, as measured by the trypan blue exclusion test on day 9 (control vs. Delta4; $17.56 \pm 0.60 \times 10^5$ /mL vs. $7.13 \pm 1.32 \times 10^5$ /mL, $P < 0.0001$; Fig. 3A). This effect on cellular proliferation was also confirmed by performing an MTT assay on day 9 ECFCs (Fig. 3B). The other two ligands assayed did not have a detectable effect on cell proliferation. Furthermore, we were able to confirm that none of the four ligands examined induced apoptosis of ECFCs using flow cytometry with Annexin V-FITC and phosphatidylserine staining (data not shown).

We examined the effect of immobilized Delta4 on colony-forming capacity of ECFCs (Fig. 3C). Immobilized Delta4 did not reduce colony-forming capacity of ECFCs, despite the fact that Delta4 has been shown to act as an inhibitor of proliferation in other contexts. Moreover, ECFCs incubated with

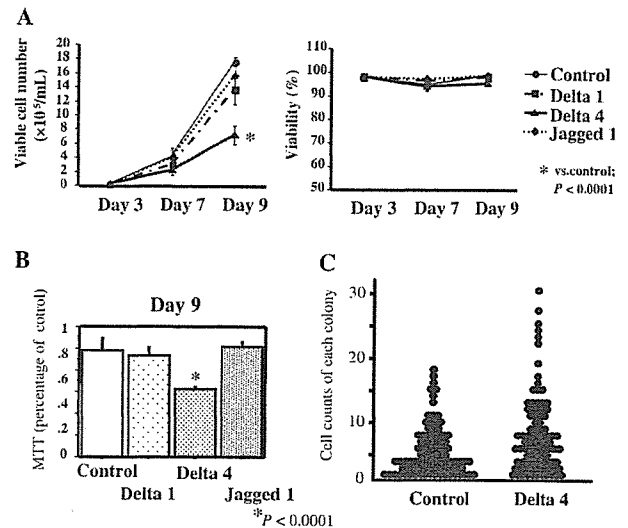


Fig. 3. Effects of immobilized Notch ligands on proliferation of erythroid colony forming cells (ECFCs). Day 3 ECFCs (1.0×10^4 /mL) were incubated with or without immobilized Notch ligands (30 mg/mL), and (A) viable cell number (left panel) and cell viability (right panel) were measured by trypan blue exclusion at the indicated days. (B) Measurement of cell growth by MTT assay. (C) Effect of immobilized Delta4 on function of erythroid colony formation. Each point shows the number of hemoglobinized cells in randomly selected colonies. Data are representative of two experiments.

immobilized Delta4 tended to form erythroid colonies that are larger than control colonies.

Expression of HES-1 mRNA increases after stimulation with immobilized Delta4

To investigate if Delta4 actively induces Notch related intracellular signaling in erythroid progenitor cells, we determined the ratio between the number of copies of mRNA of HES-1 (a Notch target gene) and the number of copies of 18S rRNA (an internal control) on days 6 and 9 using real-time PCR. The relative expression of HES-1/number of copies of 18S in the culture with ligand was then compared with the control. Increased expression of HES-1 mRNA became evident after stimulation with immobilized Delta4 on day 6 ($P = 0.0003$; Fig. 4A). In contrast, no significant change in expression of HES-1 could be detected when cells were stimulated by non-immobilized, soluble Delta4-FLAG fusion protein (10 mg/mL; Fig. 4B).

Soluble Delta4 slightly inhibits maturation of ECFCs

We next investigated the effects of soluble Delta4 on maturation of ECFCs by using flow cytometry to determine the level of expression of TfR and GPA. When day 3 ECFCs were cultured with soluble Delta4 (10 mg/mL) at a low cell concentra-

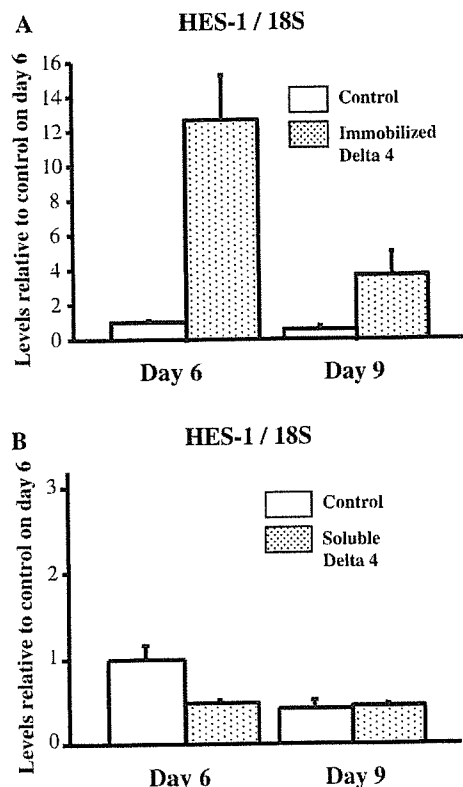


Fig. 4. Induction of the Notch target gene, hairy enhancer of split (HES)-1, by Delta4. Relative expression of mRNA of the Notch target gene, HES-1, and the number of copies of 18S rRNA (a ubiquitously expressed gene) were determined by real-time PCR on days 6 and 9 for cells cultured with IgG1-kappa (white bar), immobilized Delta4 (gray bar) or soluble Delta4 (gray bar). Relative expression of hairy enhancer of split (HES)-1/number of copies of 18S in the culture with ligand was compared with the control. (A) Expression of HES-1 mRNA increases after stimulation with immobilized Delta4. (B) Expression of HES-1 after stimulation by soluble Delta4.

tion, cell-cell interaction among ECFCs is likely to be at negligible levels. After 6 d of culture, the percentage of GPA+ cells was lower than that in the control (63.7% vs. 52.1%, Fig. 5A, left panel). The percentage of TfR+/GPA+ cells also decreased in cultures with soluble Delta4 (64.3% vs. 53.1%, Fig. 5A, right panel). However, this effect of soluble Delta4 was less significant than that seen in cells cultured with immobilized Delta4 (Fig. 2B). This finding is consistent with the expression data for HES-1 mRNA (Fig. 4).

In order to ask if soluble Delta4 inhibited maturation of ECFCs in cultures at a high density of day 3 ECFCs, in which endogenous cell-cell interaction among ECFCs might exist, we cultured day 3 ECFCs with soluble Delta4 (10 mg/mL) at a high density (1.0×10^5 /mL). Unexpectedly, the percentage of GPA+ cells was higher than that seen in the control (46.6% vs. 65.6%, Fig. 5B, left panel). In addition, the percentage of TfR+/

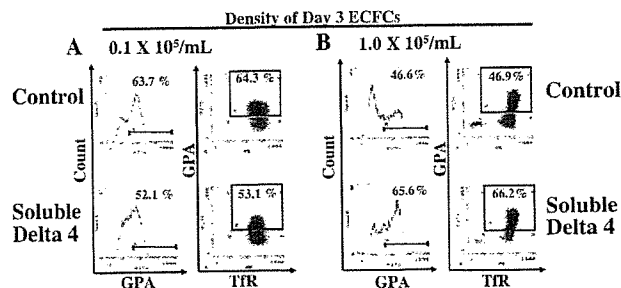


Fig. 5. Effects of non-immobilized Delta4 on erythroid maturation. Day 3 erythroid colony forming cells (ECFCs) were incubated at low density (A; 0.1×10^5 /mL) or at high density (B; 1.0×10^5 /mL) with or without soluble Delta4 (10 mg/mL), and expression of transferrin receptor and glycoprotein A (GPA) on day 9 ECFCs were determined by flow cytometry. In the left panel of each figure, numbers in corners represent the percentage of GPA+ cells. In the right panel of each figure, numbers in corners represent the percentage of gated events within that quadrant. Data are representative of three experiments.

GPA+ cells significantly increased with Delta4 treatment (46.9% vs. 66.2%, Fig. 5B, right panel). These results suggested that maturation of ECFCs could be promoted by the addition of soluble Delta4 to the culture medium when the cells are at high density. In contrast, immobilized Delta4 inhibited maturation of ECFCs plated at high cell density (1.0×10^5 /mL; data not shown).

Effects of soluble Delta4 differ with changes in cell density

To further examine the effects of cell-cell interaction in modulation of erythroid maturation, we examined the effect of soluble Delta4 in cells at different densities at day 3 (Fig. 6A). In each of four independent experiments, at low cell-density, the percentage of TfR+/GPA+ cells decreased as compared with controls with addition of soluble Delta4. When cell density of the culture was increased, the effect of soluble Delta4 was lost, and finally, at high cell density, the percentage of TfR+/GPA+ cells was higher with soluble Delta4 than that seen in the control. Cumulative data are shown in Fig. 6B. The percentage of TfR+/GPA+ cells was significantly lower than the control at low cell density (0.25×10^5 /mL, $P = 0.02$) and higher at high cell density (1.0×10^5 /mL, $P = 0.03$).

Discussion

Notch signaling maintains an immature, precursor state of cells and affects the lineage outcome in a number of developmental and differentiation systems (15–18, 25–29). When progenitors have both Notch receptors and ligands, Notch receptors on one cell are activated by ligands on neighboring cells. This results in inhibition of differentiation of

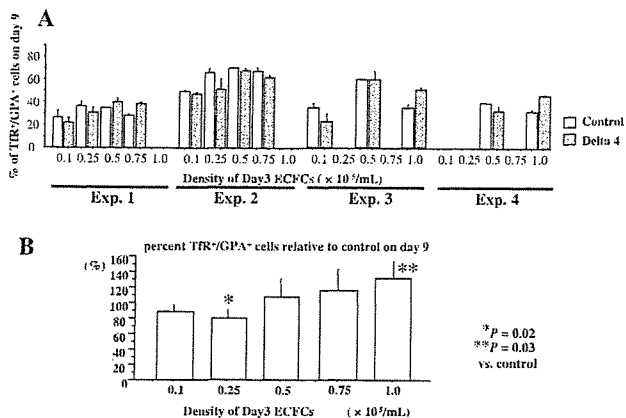


Fig. 6. Effect of soluble Delta4 on maturation at different cell densities. Day 3 erythroid colony forming cells were incubated at various cellular densities (0.1, 0.25, 0.5, 0.75, 1.0×10^5 /mL) in serum-free liquid medium with or without soluble Delta4 for 6 d. (A) The percentage of transferrin receptor (TFR)+/glycophorin A (GPA)+ cells on day 9 was determined in four independent experiments. (B) Each bar represents the ratio of TFR+/GPA+ cell number in soluble Delta4-treated cultures to the number of double-positive cells in the control cultures at each cellular density.

receptor-expressing cells at the same time that ligand-expressing cells differentiate along a fate-specific pathway. The result is that the two cell populations exist in different stages of differentiation as a result of the effects of differential Notch signaling.

In studies of hematopoietic cells, Notch signaling has mainly been investigated in multipotent hematopoietic stem cells. With regard to the effect of Notch signaling on commitment to an erythroid lineage fate, activation of Notch signaling inhibits differentiation along erythroid lineage in mouse stem cells (18, 30). Moreover, in human and mouse erythroleukemic cells, Notch signaling inhibits erythroid differentiation (31, 32). Despite these advances in our understanding, the role of Notch signaling in committed hematopoietic progenitor cells has not been fully explored.

Here, we have shown that Notch receptors and their ligands are expressed in human erythroid progenitor cells purified from peripheral blood (ECFCs). In addition, we found that Notch signaling through immobilized Delta4 inhibits erythroid maturation and proliferation without affecting their function or inducing apoptosis.

The level of the expression patterns of Notch receptors and ligands on ECFCs differs between cells at different stages of maturation, as seen in the B-cell lineage (33). These observations suggest the possibility that the pattern of functional Notch receptors and ligands differs at different stage of maturation of ECFCs.

We detected a maturation inhibition effect of Notch signaling by comparing the percentage of

TFR+ and GPA+ cells. The effect of Notch signaling on ECFCs was rather faint and thus difficult to detect by less sensitive methods, such as benzidine staining (data not shown). Kumano *et al.* reported that Notch1 signaling inhibited erythroid differentiation in mouse erythroleukemic cell lines F5-5 and that the expression of GATA-2, one of the transcription factors, which expression level decreased during erythroid differentiation, was sustained after Notch1 activation (34). However, it has not been known if any transcription factors relevant to erythroid differentiation, such as GATA-2, were affected by activation of Notch signaling in primary human hematopoietic progenitor cells. Although we analyzed the expression level of GATA-2 mRNA by real-time PCR, we found no significant difference between control cells and cells stimulated by immobilized Delta4. GATA-2 is known to be important in decision of lineage commitment of multipotent progenitor cells toward erythroid lineage and in primary progenitor cells Notch may play a role at more differentiated stage (i.e., at a stage where GATA-2 mRNA level has already decreased).

We show that immobilized Delta4 induced elevation of HES-1 mRNA expression in ECFCs, a cell type in which Delta4 also delayed maturation. Ishiko *et al.* reported that forced expression of HES-1 inhibited erythroid differentiation in an erythroid/megakaryocytic cell line (K562) and in murine normal hematopoietic stem/progenitor cells. Thus, in human erythroid progenitor cells, similar HES-1-directed mechanisms may also take part in Notch signaling.

The observation that ECFCs expressed Notch and Delta4, together with the observation that immobilized Delta4 delayed erythroid maturation, indicate that erythroid progenitors may modulate maturation of one another via cell-cell interaction. Moreover, the data also suggest that interaction occurs between ECFCs and endothelial cells. In vertebrates, some of Notch receptors and ligands are expressed in the vascular endothelium (35–37). Delta4 is predominantly expressed in arterial endothelium (10). Recently, it was shown that Delta4 can function as a ligand for Notch4 in a human primary endothelial cell line derived from neonatal dermal microvasculature (38). It is possible that ECFCs stimulate Notch signaling in primary endothelial cells through Delta4-Notch binding. It was also reported that VEGF induces expression of Notch1 and of its ligand, Delta-like 4, in human arterial endothelial cells (39). Given that ECFCs produce VEGF (data not shown), and given that VEGF stimulates the proliferation of endothelial cells, negative feedback via Delta-like 4-Notch binding may occur between ECFCs and endothelial cells.

Our study shows that the elevation of HES-1 mRNA expression and inhibition of maturation of ECFCs induced by soluble Delta4 are less prominent than what is seen with immobilized Delta4. Similar results were obtained using immobilized or soluble Delta1 in C2 myoblasts and U2OS cells. In these cell lines, Notch is not activated by soluble Delta1. Furthermore, soluble Delta1 blocks the effect of immobilized Delta1 (40). These results imply that ligation of Notch receptor by a stabilized ligand may be essential for full activation of Notch signaling.

Surprisingly, effects of soluble Delta4 differ with changes in cell density. When the density of day 3 ECFCs is high, there may exist many cell-cell interactions among ECFCs and thus, signaling between the Notch receptor on one ECFC and the endogenous Notch ligand on neighboring ECFCs may occur. When soluble Delta4 is added to day 3 ECFCs, some of the soluble Delta4 (but not the endogenous Delta4) binds to Notch receptors. Consistent with the idea that the effect of soluble Delta4 is smaller than that of endogenous Delta4, inhibitory effect of Notch signaling on maturation is less prominent than that seen in the culture without soluble Delta4. On the other hand, when the density of day 3 ECFCs is low, the frequency of cell-cell interaction among ECFCs is rare and thus, we expect that signaling between endogenous Notch receptors and ligands will only rarely be seen. But when soluble Delta4 is added to this culture, soluble Delta4 can bind to Notch receptors on ECFCs and the ligand weakly inhibits the maturation of receptor-expressing cells. The result is that in the presence of soluble Delta4, maturation is inhibited slightly in comparison with what is seen in cells without soluble Delta4. Based on these observations, we conclude that there exists a cell-cell interaction among ECFCs that acts via the Notch signal transduction pathway.

In conclusion, we demonstrate that Notch signaling plays a pivotal role in the regulation of erythroid maturation and proliferation. Additionally, the data suggest that cell-cell interactions modulate growth of erythroid progenitor cells via the Notch pathway. However, the precise molecular mechanism(s) by which Notch signaling modulates erythroid maturation is not fully understood. Further investigation of the mechanisms of inhibition of erythroid progenitor cell maturation by Notch signaling components may be revealing.

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Genetic polymorphism of cholesterol 7 α -hydroxylase (CYP7A1) and colorectal adenomas: Self Defense Forces Health Study

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Bile acids have long been implicated in colorectal carcinogenesis, but epidemiological evidence is limited. Cholesterol 7 α -hydroxylase (CYP7A1) is the rate-limiting enzyme producing bile acids from cholesterol. A recent case-control study showed a decreased risk of proximal colon cancer associated with the CC genotype of the CYP7A1 A-203C polymorphism. The present study examined the relationship between the CYP7A1 A-203C polymorphism and colorectal adenoma, which is a well-established precursor lesion of colorectal cancer. The study subjects comprised 446 cases of colorectal adenomas and 914 controls of normal total colonoscopy among men receiving a preretirement health examination at two hospitals of the Self Defense Forces (SDF). The CYP7A1 genotype was determined by the polymerase chain reaction–restriction fragment length polymorphism method. Statistical adjustment was made for age, hospital, rank in the SDF, smoking, alcohol use, body mass index, physical activity and parental history of colorectal cancer. The CYP7A1 polymorphism was not measurably related to the overall risk of colorectal adenomas. However, the CC genotype was associated with a decreased risk of proximal colon adenomas, but not of distal colon and rectal adenomas. Adjusted odds ratios of proximal colon adenomas (95% confidence intervals) for the AC and CC genotype versus AA genotype were 0.82 (0.54–1.24) and 0.56 (0.34–0.95), respectively. The findings add to evidence for the role of bile acids in colorectal carcinogenesis. The CC genotype of the CYP7A1 A-203C polymorphism probably renders lower activity of the enzyme synthesizing bile acids. (*Cancer Sci* 2006; 97: 406–410)

Bile acids have long been implicated in colorectal carcinogenesis.⁽¹⁾ Primary bile acids, such as cholic and chenodeoxycholic acids, are produced from cholesterol in the liver, and more than 95% of those passing through the ileum are reabsorbed and return to the liver. Secondary bile acids, mainly deoxycholic and lithocholic acids, are formed by the anaerobic bacterial flora in the large bowel from primary bile acids that escape absorption in the ileum.⁽¹⁾ Secondary bile acids are known to promote colorectal carcinogenesis in animals,^(2,3) and molecular mechanisms have been found regarding the effect of bile acids promoting colorectal carcinogenesis.^(4,5) However, epidemiological evidence remains elusive regarding the relationship between bile acids and

colorectal cancer. Fecal concentrations of secondary bile acids are higher in populations at high risk of colorectal cancer.^(6,7) Several case-control studies have shown that fecal or serum levels of secondary bile acids are higher in patients with colorectal cancer or adenoma than in those without these lesions.^(8–11) A high ratio of serum deoxycholic acid to cholic acid tended to be associated with an increased risk of colorectal cancer in a prospective study.⁽¹²⁾

Cholesterol 7 α -hydroxylase (CYP7A1) is the rate-limiting enzyme that converts cholesterol into cholesterol 7 α -hydroxycholesterol in the first step of the classical pathway of bile acid synthesis.⁽¹³⁾ Overexpression of cholesterol 7 α -hydroxylase activity in hamsters results in a dose-dependent decrease in plasma cholesterol concentrations.⁽¹⁴⁾ In mice deficient in cholesterol 7 α -hydroxylase, fecal excretion of bile acids as well as the bile acid pool is decreased.⁽¹⁵⁾ In humans, a polymorphism in the promoter region of the CYP7A1 gene (CYP7A1 A-203C) is associated with plasma concentrations of total or low density lipoprotein (LDL) cholesterol, suggesting lower enzyme activities in those with the -203CC genotype,^(16,17) although this finding was not replicated in another study.⁽¹⁸⁾ The CYP7A1 A-203C polymorphism may modulate transcription of the CYP7A1 gene and consequently the rate of bile acid synthesis.

In a case-control study of colorectal cancer,⁽¹⁹⁾ individuals with the CYP7A1-203CC genotype were associated with a lower risk of proximal colon cancer, but not of distal colon and rectal cancer. In the present study, we examined the relationship between the CYP7A1 A-203C polymorphism and colorectal adenomas, which is a well-established precursor lesion of colorectal cancer.^(20,21)

Materials and Methods

Subjects

Study subjects were male self-defense officials who received a preretirement health examination at the Self Defense

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Forces (SDF) Fukuoka or Kumamoto Hospital from January 1997 to March 2001. The preretirement health examination is a program offering comprehensive medical examination for those retiring from the SDF. Details of the health examination and a lifestyle survey have been described elsewhere.^(22,23) In addition to blood samples for routine use in the health examination, a sample of 7 mL fasting venous blood was obtained for the purpose of medical research with written informed consent. The study was approved by the ethical committee of Kyushu University.

The study subjects comprised 446 cases of colorectal adenomas and 914 controls of normal total colonoscopy. In the consecutive series of 2454 men, except for eight who refused to participate in the survey, 2377 (97%) underwent total or partial colonoscopy. We excluded 242 men with a prior history of colectomy ($n = 17$), colorectal polypectomy ($n = 212$), malignant neoplasm ($n = 27$) or inflammatory bowel disease ($n = 1$). Numbers of men according to the macroscopic findings of colonoscopy were: normal 1073, colorectal polyps 938, carcinoma one and other non-polyp benign lesions 123. Of the 938 with colorectal polyps, 461 were found to have at least one histologically confirmed adenoma without *in situ* or invasive carcinoma. Of the 1073 men with a normal result, 949 underwent total colonoscopy and were used as controls subjects. Of these cases and controls, DNA was not available for six cases and 13 controls, and genotyping was unsuccessful with nine cases and 22 controls, thus the remaining 446 cases and 914 controls were used in the analysis.

Numbers of adenoma cases by subsite were: proximal colon only 146, distal colon only 180, rectum only 42, and multiple sites 78. Proximal colon included cecum, ascending colon and transverse colon. Cases of adenoma with sizes of <5 mm, 5–10 mm and ≥ 10 mm (the largest size for multiple adenomas) numbered 260, 149 and 37, respectively.

Genotyping

DNA was extracted from buffy coat stored at -80°C by using a commercial kit (QIAGEN, Hilden, German). The *CYP7A1* genotype was determined by the polymerase chain reaction (PCR)–restriction fragment length polymorphism method, as described previously by Han *et al.*,⁽¹⁸⁾ using primers 5'-AATGT TTTTC CAGT TCTCT TTC-3' (sense) and 5'-AATTA GCCAT TTGTT CATTC TATTA G-3' (antisense). The PCR was carried out in a reaction mixture of 10 μL containing 0.5 IU of *Taq* and 1 μL of template DNA with a concentration of approximately 50–150 ng/ μL . After the initial denaturation at 94°C for 4 min, 30 cycles of PCR were carried out for 30 s at 94°C , 30 s at 53°C and 30 s at 72°C , with a final extension at 72°C for 7 min. The PCR product of 393 bp was digested with 10 IU of *BsaI* in a reaction mixture of 20 μL for 3 h at 50°C . The digestion resulted in fragments of 300 and 93 bp for the *A* allele, and fragments of 261, 93 and 39 bp for the *C* allele. The digested fragments were electrophoresed on a 3% agarose gel (NuSieve GTG), and visualized using ethidium bromide. The polymorphism was described as A-204C by Couture *et al.*,⁽¹⁷⁾ but we confirmed, by sequencing, the actual site of the polymorphism at 203 bp upstream of the transcription start site. Genotyping was carried out with case and control status unknown.

Lifestyle factors

Height and bodyweight were recorded, and body mass index (kg/m^2) was calculated. Body mass index was categorized by using the 30th, 60th and 90th percentiles in the distribution of the controls. A self-administered questionnaire ascertained smoking habit, alcohol use, leisure-time physical activity and other lifestyle factors. Lifetime exposure to cigarette smoking was expressed as cigarette-years (the average number of cigarettes smoked per day multiplied by years of smoking), and classified into four levels of 0, 1–399, 400–799 and ≥ 800 cigarette-years. Alcohol drinking was defined as having drunk alcoholic beverages at least once per week for 1 year or longer, and former alcohol use was separated from lifetime non-use of alcohol. The amount of alcohol consumed per day was calculated for current alcohol drinkers on the basis of consumption frequencies and amounts per occasion of five types of alcoholic beverages on average in the past year. Alcohol use was categorized into never, former and current use with consumption of <30, 30–59, or ≥ 60 mL of ethanol per day.

Questions on leisure-time physical activity were slightly changed in April 1999. In the earlier version, subjects were first asked about the frequency of regular participation in exercise and sport during leisure time on average in the past year using a closed-ended question (none, 1–2, 3–4, 5–6 times per week and daily). If the subjects participated in recreational physical activity at least once per week, they reported type of activity and time spent per occasion regarding at most three types of regular exercise. In the revised questionnaire, the subjects were first asked whether they participated in recreational activity regularly (one or more times per week) in the past year. Those with a regular participation reported at most three types of physical activities together with frequency per week and time spent per occasion for each activity. Type of physical activity was classified into light, moderate, heavy or very heavy activity in terms of metabolic equivalent (MET).⁽²⁴⁾ The time spent in recreational exercise was multiplied by the corresponding MET value (light 2, moderate 4, heavy 6 and very heavy 8) to yield a MET-hour score per week. Individuals were classified into four groups with the quartiles in the control group as cut-off points. Parental history of colorectal cancer was also elicited.

Statistical analysis

The association between the *CYP7A1* polymorphism and colorectal adenomas was assessed by means of adjusted odds ratio (OR) and 95% confidence interval (CI), which were derived from multiple logistic regression analysis. Statistical adjustment was made for age (continuous variable), hospital, rank of the SDF (low, intermediate and high), cigarette smoking, alcohol use, body mass index, physical activity and parental colorectal cancer. Interactions of the *CYP7A1* polymorphism with selected lifestyle factors were evaluated by the likelihood ratio test. Statistical significance was declared if a two-sided *P*-value was less than 0.05 or if the 95% CI did not include unity. All statistical analyses were carried out using SAS version 8.2 (SAS Institute, Cary, NC, USA).

Results

The characteristics of colorectal adenoma cases and controls are summarized in Table 1. The age ranges were 50–57 years in the cases and 47–59 years in the controls, but the mean ages were identical in the two groups. Cigarette and alcohol consumption were greater in the cases than in the controls, and body mass index was also greater in the former group. Men with low physical activity and those with parental history of colorectal cancer were slightly more frequent in the cases.

In the cases, proportions of the AA, AC and CC genotypes were 26, 50 and 25%, respectively (Table 2). The corresponding proportions in the control group were 24, 49, and

Table 1. Characteristics of colorectal adenoma cases and controls

Variable	Cases (n = 446)	Controls (n = 914)	P-value†
Age (years), mean (SD)	52.4 (0.83)	52.4 (0.92)	0.82
Hospital (%)			
Fukuoka	71.1	68.4	0.31
Kumamoto	28.9	31.6	
Rank (%)			
Low	60.3	62.3	0.68
Intermediate	25.3	23.2	
High	14.3	14.6	
Cigarette-years (%)			
0	21.3	34.1	<0.0001
1–399	14.3	19.1	
400–799	44.8	33.8	
≥800	19.5	12.9	
Alcohol use (%)			
None	11.2	14.6	0.0002
Past	2.9	3.1	
<30 (mL/day)	21.5	31.0	
30–59	34.1	28.8	
≥60	30.3	22.6	
BMI (kg/m ²), mean (SD)	24.1 (2.79)	23.7 (2.46)	
MET-hours/week (%)			
<5	28.5	23.9	0.32
5–14	23.8	25.9	
15–24	24.2	24.9	
≥25	23.5	25.3	
Parental CRC (%)			
Negative‡	95.3	96.6	0.23
Positive	4.7	3.4	

†Based on t-test or χ^2 -test. ‡Including two cases and two controls with parental colorectal cancer (CRC) unknown. BMI, body mass index; MET, metabolic equivalent; SD, standard deviation.

Table 2. CYP7A1 A-203C polymorphism and risk of colorectal adenoma

Genotype	Cases		Controls		Crude OR	Adjusted OR (95% CI)†
	n	%	n	%		
AA	115	25.8	219	24.0	1.00	1.00 (referent)
AC	221	49.6	452	49.5	0.93	0.93 (0.70–1.24)
CC	110	24.7	243	26.6	0.86	0.87 (0.62–1.20)

†Adjusted for age, hospital, rank, body mass index, cigarette smoking, alcohol use, physical activity and parental history of colorectal cancer. CI, confidence interval; OR, odds ratio.

27%, respectively. These frequencies were in agreement with the Hardy–Weinberg equilibrium ($P = 0.98$ for cases and $P = 0.95$ for controls). Overall, the CYP7A1 polymorphism was not measurably associated with colorectal adenomas, although the OR for the CC genotype versus AA genotype was slightly lower than unity.

When the association with the CYP7A1 polymorphism was examined for adenomas of the proximal colon, distal colon and rectum separately, the OR of proximal colon adenomas showed a statistically significant decrease among individuals with the CC genotype compared with those with the AA genotype. The CYP7A1 polymorphism was unrelated to adenomas at the distal colon and rectum (Table 3).

Adjusted OR of small colorectal adenomas (<5 mm) for the AC and CC genotypes versus AA genotype were 1.11 (95% CI 0.78–1.58) and 0.87 (95% CI 0.58–1.32), respectively. The corresponding OR of large colorectal adenomas (≥5 mm) were 0.74 (95% CI 0.50–1.09) and 0.86 (95% CI 0.55–1.33), respectively. Adjusted OR of small proximal colon adenomas ($n = 86$) were 1.09 (95% CI 0.64–1.86) for the AC genotype and 0.41 (95% CI 0.20–0.87) for the CC genotype compared with the AA genotype, and adjusted OR of large proximal colon adenomas ($n = 60$) for the AC and CC genotypes were 0.52 (95% CI 0.28–0.98) and 0.71 (95% CI 0.36–1.41), respectively.

Finally, we explored interactions between the CYP7A1 polymorphism (three genotypes) and lifestyle factors for the risk of proximal colon adenomas with two categories used for smoking (<400 cigarette-years vs ≥400 cigarette-years), alcohol use (<30 mL/day including past alcohol use versus ≥30 mL/day), body mass index (<25.0 kg/m² vs ≥25.0 kg/m²), and physical activity (<15 MET-hours/week vs ≥15 MET-hours/week). There was no measurable interaction for any of the lifestyle factors under study: smoking ($P = 0.38$), alcohol use ($P = 0.93$), body mass index ($P = 0.62$) and physical activity ($P = 0.39$).

Table 3. CYP7A1 A-203C polymorphism and risk of colorectal adenoma by location

Genotype	Proximal colon		Distal colon		Rectum	
	n†	OR (95% CI)‡	n†	OR (95% CI)‡	n†	OR (95% CI)‡
AA	44/219	1.00 (referent)	42/219	1.00 (referent)	10/219	1.00 (referent)
AC	73/452	0.82 (0.54–1.24)	86/452	1.01 (0.67–1.52)	23/452	1.07 (0.49–2.35)
CC	29/243	0.56 (0.34–0.95)	52/243	1.14 (0.72–1.80)	9/243	0.89 (0.35–2.31)

†Numbers of cases/controls. ‡Adjusted for age, hospital, rank, body mass index, cigarette smoking, alcohol use, physical activity and parental history of colorectal cancer. CI, confidence interval; OR, odds ratio.

Discussion

The present study was the first that examined the relationship between a functional *CYP7A1* polymorphism and colorectal adenomas, and showed a statistically significant decrease in the risk of proximal colon adenomas, but not of distal colon and rectal adenomas, associated with the *CC* genotype of the *CYP7A1 A-203C* polymorphism. The findings are consistent with the recent observation in Japan that the *CC* genotype of this polymorphism is related to a decreased risk of proximal colon cancer exclusively,⁽¹⁹⁾ and provide further evidence for the role of bile acids in colorectal carcinogenesis. Although the relationship between the *CYP7A1 A-203C* polymorphism and fecal or serum bile acids has not been investigated directly, individuals with the *CC* genotype probably have lower exposure to bile acids due to decreased activity of 7 α -hydroxylase.⁽²⁵⁾ However, further studies are required to assess functionality of the *CYP7A1 A-203C* polymorphism. It is possible that this polymorphism does not directly modulate the gene expression. Another linked polymorphism in the *CYP7A1* gene may be of functional relevance to bile acid production.

The *CYP7A1 A-203C* polymorphism was first identified as a genetic determinant of plasma total and LDL cholesterol levels through sequential steps of sibling-pair linkage analysis, DNA sequencing and association studies within families and in unrelated individuals in the USA.⁽¹⁶⁾ In that study, the *CC* homozygotes had higher levels of total and LDL cholesterol in plasma than the *AA* homozygotes. A supportive finding was reported for men but not for women in the Framingham Offspring Study.⁽¹⁷⁾ In contrast, a study of Micronesian islanders showed no association between the *CYP7A1 A-203C* polymorphism and serum apolipoprotein B, a surrogate of LDL cholesterol.⁽¹⁸⁾ In this regard, it is of interest whether serum cholesterol levels differ by the polymorphism in the present study subjects. A preliminary analysis showed no measurable variation in serum total cholesterol levels according to the *CYP7A1 A-203C* polymorphism; in the whole subjects excluding men under lipid-lowering medication (20 cases and 41 controls), adjusted means (and standard errors) of fasting total cholesterol concentrations for the *AA*, *AC* and *CC* genotypes were 203.1 (1.9), 202.5 (1.3) and 203.2 (1.8) mg/dL, respectively, after controlling for age, hospital, rank, smoking, alcohol use, body mass index and physical activity. This lack of association is not necessarily surprising because many other factors, including dietary fat, influence the between-individual variation in serum total and LDL cholesterol. Different dietary habits may have masked the genotype–lipid association in individuals.

Several lines of epidemiological evidence have suggested that bile acids may increase the risk of proximal colon cancer selectively. Patients with the gallbladder removed have an increased risk of proximal colon cancer.^(26–28) Cholecystectomy results in increased fecal excretion of secondary bile acids

due to an increase in the bile acid pool in the enterohepatic circulation and increased degradation of primary bile acids.^(29,30)

Low concentrations of serum total or LDL cholesterol have been related to increased risk of colon cancer in many prospective studies.⁽³¹⁾ Whereas this inverse association has generally been ascribed to the effect of preclinical cancer existing at the baseline,⁽³¹⁾ an increased risk of proximal colon cancer associated with low cholesterol levels persisted 10–20 years later in a prospective study of Japanese people in Hawaii.⁽³²⁾ In a case-control study, lower levels of serum total and LDL cholesterol were observed in cases of proximal colon cancer alone than in controls.⁽³³⁾ In that study, individuals with the *E4* allele for apolipoprotein E, in whom bile acid synthesis is decreased,⁽³⁴⁾ had a lower risk of proximal colon cancer and adenomas.

The present study had methodological advantages in that colonoscopy was carried out non-selectively in a defined population and in that the absence of polyp lesions was confirmed in the control subjects by total colonoscopy. The study subjects were not representative of Japanese men in the general population, but selection was unlikely to exist with regard to the genetic polymorphism under study. The allele frequency of *CYP7A1-203C* (51%) in the control subjects in the present study was quite similar to that observed in a random sample of adult residents in an area in Japan (50%),⁽¹⁹⁾ whereas these values are slightly greater than those reported in Caucasians (approximately 40%).^(16,17) Lack of dietary information was a weakness in the present study. High-fat diet increases bile acid excretion,^(35,36) and thus the interaction between the *CYP7A1 A-203C* polymorphism and fat intake deserves further studies. Another weakness was the small number in the subgroup analysis, especially regarding size-specific risks of proximal colon adenomas. Decreased risk of proximal colon adenomas associated with the *CC* genotype did not seem to differ by size, but the observed OR were variable. Moreover, it was uncertain whether the risk of proximal colon adenomas was decreased in individuals with the heterozygous *AC* genotype. Larger studies are required to address these questions conclusively.

In conclusion, a case-control study demonstrated a decreased risk of proximal colon adenoma in individuals with the *CC* genotype of the *CYP7A1 A-203C* polymorphism, which probably renders lower activity of the enzyme synthesizing bile acids. The findings add to evidence for the role of bile acids in colorectal carcinogenesis.

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Relation of alcohol use and smoking to glucose tolerance status in Japanese men[☆]

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Abstract

To investigate the relation of alcohol use and cigarette smoking to glucose tolerance status, we performed a cross-sectional study of 3038 male officials aged 46–59 years in the Self-Defense Forces. Glucose tolerance status was determined by a 75-g oral glucose tolerance test. A self-administered questionnaire was used to ascertain alcohol use, smoking habits, and other lifestyle characteristics. Statistical adjustment was made for parental history of diabetes, body mass index, and leisure-time physical activity. Alcohol use was positively associated with impaired fasting glucose, impaired glucose tolerance, and type 2 diabetes mellitus each. The association was dose-dependent, with odds of each category of glucose intolerance increased even among those with moderate alcohol use. Cigarette smoking was not related to any categories of glucose intolerance. Alcohol use may confer increased risks not only of type 2 diabetes mellitus but also of preceding glucose intolerance status. Smoking does not seem to deteriorate glucose tolerance.

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1. Introduction

The prevalence of type 2 diabetes mellitus has increased and is becoming the great public health problem in many parts of the world [1]. Obesity and physical inactivity are well known to increase the risk of

type 2 diabetes mellitus [2], but other factors associated with type 2 diabetes mellitus remain uncertain. Alcohol use has been a matter of interest in the epidemiology of type 2 diabetes mellitus. Moderate alcohol use was shown to be related to increased insulin sensitivity in several [3,4], but not all [5,6], studies. Epidemiologic findings are inconsistent regarding the relation between alcohol use and type 2 diabetes mellitus. While several prospective studies showed a protective association between moderate alcohol use and type 2 diabetes mellitus [7–11], others failed to find such an association [12,13]. Some studies observed an increased risk of type 2 diabetes mellitus associated with alcohol use [14–18].

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On the other hand, cigarette smoking was reported to be related to increased risk of type 2 diabetes mellitus in several studies [9,19–21].

Type 2 diabetes mellitus is a morbid condition of continuum, and impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) precede type 2 diabetes mellitus [22]. Not many studies have addressed the association of either alcohol use or cigarette smoking with the prediabetic conditions [23–26]. In this study, we investigated the relation of alcohol use and cigarette smoking to glucose tolerance status determined by a 75-g oral glucose tolerance test (OGTT) in middle-aged Japanese men.

2. Materials and methods

2.1. Study subjects

Subjects were male self-defense officials who received a preretirement health examination from January 1997 to March 2002 at the Self-Defense Forces (SDF) Fukuoka Hospital or the SDF Kumamoto Hospital. The preretirement health examination is a nationwide program, which offers a comprehensive medical examination for those retiring from the SDF. Details of the health examination have been described elsewhere [27,28].

In the consecutive series of 3416 men aged 46–59 years, a total of 3225 men were included in this study; 8 men refused to participate in the study, 17 men were undetermined as regards glucose tolerance status, and 166 men were excluded for the following reasons: endocrine diseases ($n = 15$), chronic pancreatitis ($n = 9$), chronic hepatitis or liver cirrhosis ($n = 78$), use of steroids ($n = 9$), past history of gastrectomy ($n = 56$), and missing information as regards covariates under study ($n = 4$); some men had two or more conditions for exclusion. Furthermore, 187 men with a history of dietary or drug treatment for type 2 diabetes mellitus were excluded irrespective of their glucose levels. Thus a total of 3038 men remained in the present analysis.

The study was approved by the ethical committee of the Kyushu University Faculty of Medical Sciences. All study subjects gave written informed consent prior to their participation in the study.

2.2. Laboratory methods

The routine medical examination included a 75-g OGTT during a 5-day admission among others. After an overnight fast, venous blood was drawn for measurement of plasma glucose before and 2 h after the oral challenge. Plasma glucose levels were assayed by the glucose oxidase method using commercial reagents (Shino Test Co. Ltd., Tokyo) at each hospital. Subjects were classified as having normal glucose tolerance, IFG, IGT, or type 2 diabetes mellitus by the World Health Organization (WHO) criteria revised in 1998 [22].

Body weight and height were recorded, and body mass index (BMI) (kg/m^2) was calculated as a measure of obesity.

2.3. Questionnaire

A self-administered questionnaire was used to ascertain alcohol use, smoking habits, leisure-time physical activity, and other lifestyle characteristics. Alcohol drinkers were defined as those who had drunk once a week or more over a period of 1 year or longer. Past drinkers were separated from lifelong nondrinkers. Current drinkers were asked about frequency and amount of consumption per occasion of five different alcoholic beverages (*sake*, *shochu*, beer, whiskey/brandy, and wine) on average in the past year, and their alcohol intake was estimated using the approximate volume concentration of alcohol in each beverage. The estimated intake of alcohol was found to be highly correlated with the intake based on the 28-day diet record in the past year; Spearman rank correlation coefficient was 0.91 [29]. Ever smokers were defined as those who had smoked one cigarette or more per day over a period of 1 year or longer. Past smokers were separated from never smokers. Current and past smokers reported the average number of cigarettes smoked per day and the total years of smoking.

Questions on leisure-time physical activity were slightly changed in April 1999. Before the revision, the subjects were first asked about the frequency on average in the past year of regular participation in recreational exercise and sport using a closed ended question (none, 1 to 2, 3 to 4, 5 to 6 times per week and daily). If the subjects participated in recreational physical activity once per week or more frequently, they reported the type of regular exercise or sport and the time spent per occasion. Up to three types of regular exercises were recorded. In the revised questionnaire, the subjects were first asked whether they participated in recreational activity regularly (one or more times per week) in the past year. Those with a regular participation reported up to three types of physical activities together with frequency per week and time spent per occasion for each activity. Reported type of exercise and sport was classified into light, moderate, heavy, or very heavy activity in accordance with the published energy expenditure requirements in terms of metabolic equivalent (MET) for different physical activities [30]. The time spent in recreational exercise was multiplied by the corresponding MET value (light 2, moderate 4, heavy 6, and very heavy 8) to yield an MET-hour score.

2.4. Statistical analysis

Multiple logistic regression analysis was used to examine the relation of alcohol use and cigarette smoking to glucose tolerance status in terms of odds ratios (ORs) of individual categories of glucose intolerance. Ordinal logistic regression was also applied with ordinal values (0, 1, 2, and 3) assigned to normal glucose tolerance, IFG, IGT, and diabetes, respectively. Statistical adjustment was made for hospital, rank in the