Transcriptional Induction of Nur77 by Indomethacin That Results in Apoptosis of Colon Cancer Cells

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Non-steroidal anti-inflammatory drugs (NSAIDs) have cancer preventive and tumor regressive effects in the human colon, perhaps due to their capability to induce apoptosis of the colon cancer cells. Here, we report that indomethacin induced the expression of Nur77 which has been implicated in activation-induced apoptosis of T-lymphocytes, in a colon cancer cell line, HCT-15. The transcript- and protein-level, the transcriptional activity of Nur77 promoter, and the DNA binding of Nur77 were significantly induced following indomethacin treatment. Among the two potential *trans*-acting factors that activate Nur77-promoter, indomethacin induced DNA binding and reporter gene activity of AP-1, but not that of related serum response factor (RSRF), suggesting that the transcriptional induction of Nur77 may be mediated through activation of AP-1. Further, we showed that all-*trans*-RA repressed the induction of Nur77 as well as the apoptosis-induced by indomethacin, providing evidence that transcriptional induction of Nur77 may be an important mechanism by which indomethacin induces apoptosis in colon cancer cells.

Key words Nur77; indomethacin; colon cancer; apoptosis

The non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and salicylic acid are effective in chemoprevention and chemotherapy in human colon cancer. Epidemiological studies have revealed that NSAIDs reduced the incidence of colon cancer and mortality from the disease.[1,2] Further, sulindac, an NSAID, is the first pharmacological agent demonstrated to induce regression of colonic polyps in familial adematous polyposis (FAP).[3,4] Perhaps the anti-neoplastic effects of NSAIDs are due to the apoptosis-inducing capability of the drugs since they have been observed to induce apoptosis in the lesion of FAP, as well as in several cancer cell lines including HCT-15 and HT-29.[5—7] However, the molecular details of the anti-tumor effects and the apoptotic process induced by NSAIDs have not been clearly illustrated.

Nur77 (also known as nerve growth factor-induced gene-B [NGFI-B], N10, TIS1 and Nak-1) is an orphan member of the steroid/thyroid receptor superfamily that is a transcriptional factor regulating gene expression positively or negatively.[8—10] Nur77 is an immediate-early response gene of which expression is rapidly induced by a variety of stimuli, such as treatment with growth factors and mitogens.[11—13] The involvement of Nur77 in apoptosis was first shown by its rapid induction during T-cell receptor (TCR) signaling in immature thymocytes and T-cell hybridomas. The overexpression of a dominant-negative Nur77 protein or inhibition of Nur77 expression by anti-sense Nur77 inhibited TCR-induced apoptosis, whereas the constitutive expression of Nur77 resulted in massive apoptosis, indicating that a high level induction of Nur77 gene expression plays a role in TCR-induced apoptosis.[14,15] Recently, Li *et al.* showed that treatment of lung cancer cells with 6-[3-adamantyl-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN), a novel retinoid, strongly induced apoptosis that is accompanied by a rapid induction of Nur77.[16] The strong induction of Nur77 was also observed in prostate cancer cells after stimulation with a variety of apoptosis-inducing agents,[17,18] indicating that Nur77 may be a factor that regulates apoptosis in cancer cells following treatment with chemotherapeutic drugs.

Here, we report that indomethacin remarkably induced transcriptional expression of Nur77 in a colon cancer cell line, HCT-15. Between the two potential *trans*-acting factors that activate the Nur77-promoter, *i.e.* AP-1 and related serum response factor (RSRF), indomethacin induced DNA binding and the reporter gene activity of AP-1, but not that of RSRF, suggesting that the transcriptional induction of Nur77 may be mediated through activation of AP-1. In addition, all-*trans*-retinoic acid (RA) inhibited the induction of Nur77 as well as the indomethacin-induced apoptosis of HCT-15, providing evidence that transcriptional induction of the Nur77 gene may be an important mechanism by which indomethacin induces apoptosis in colon cancer cells.

MATERIALS AND METHODS

Cell Culture and Cell Death Measurement HCT-15 (ATCC CCL 225) was obtained from the American type culture collection and maintained in minimal essential medium containing 10% fetal bovine serum. RA and indomethacin were purchased from Sigma (St. Louis, MO, U.S.A.). Cell death was measured by 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays as previously described.[19] Cells were seeded at an initial density of 10000 cells per well in 96-well plates and incubated overnight to allow cells to adhere. After treatment of cells with various concentrations of indomethacin for 12 h, the number of viable cells was determined by measuring their capacity to convert a tetrazolium salt into a blue formazan product spectrophotometrically at 570 nm.

Northern Blot Analysis Total RNA was prepared using a Qiagen RNeasy kit (Qiagen Inc., Chatsworth, CA, U.S.A.) and Northern blot analysis was performed essentially as described previously.[16] Twenty micrograms of total RNA obtained from HCT-15 cells was fractionated on a 1% agarose gel and transferred to a nylon membrane. A 1.2 Kb *Eco*RI/*Pst*I fragment of Nur77 cDNA was labeled with ^{32}P-dCTP and used as a probe. To determine that an equal amount of

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RNA was used, the expression of β-actin was examined.

Immunoprecipitation/Western Blot Analysis To detect Nur77 protein, cells were lysed in a lysis buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl, 10% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin. Five hundred μg of whole cell lysate was incubated with 2-μg anti-Nur77 antibodies (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.). The resulting immune complex was precipitated by adding 20-μl protein-G agarose slurry, washed four times with lysis buffer, subjected to 7.5% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Corp., Arlington Heights, IL, U.S.A.). The membrane was probed with the anti-Nur77 antibody (PharMingen, San Diego, CA, U.S.A.).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Total RNA was prepared using the Qiagen RNeasy kit and single-stranded DNA was synthesized from RNA in a reaction mixture containing 100 ng of random hexamer and 200 units of murine Moloney leukemia virus reverse transcriptase (GibcoBRL, Grand Island, NY, U.S.A.). PCR reaction was performed as described previously with specific primers for Nur77 (forward: 5′-CGACCCCCTGACCCCTGAGTT-3′, reverse: 5′-GCCCTCAAGGTGTTGGAGAAGT-3′), and β-actin (5′-CGTGGGCCGCCCTAGGCACCA-3′, reverse: 5′-TTGGCCTTAGGGTTCAGGGGGG-3′). The genes were analyzed under conditions in which PCR products were exponentially amplified.

Gel-Shift Assay Nuclear extract preparation and gel-shift assay were carried out as described.[20] Ten μg of nuclear extract was incubated with ^{32}P-labeled oligonucleotide for 20 min in a 20-μl reaction mixture containing 10 mM Tris buffer (pH 7.5), 100 mM KCl, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, 1 mg/ml BSA, and 5% glycerol at 25 °C for NGBI-B response elements (NBRE), AP-1-like and SP-1 binding sequences. Antibodies against Jun and Fos (Santa Cruz Biotech.) were used to show the specific binding of AP-1. The sequences of oligonucleotides used as probes in the experiments were: NBRE, 5′-GGAGTTTTAAAAGGTCATGCTCA-3′; AP-1-like sequences, 5′-GATCTCCATGCGTCACGG-3′; consensus AP-1 binding sequences, 5′-AGCTTGATGAGTC-3′; and SP-1, 5′-GATCGATCGGGGGGGGCGAG-3′.

Transient Transfection Assay The reporter genes, *i.e.*, Nur77 promoter-Luc, AP-1-RE-Luc, and RSRF-RE-Luc have been described elsewhere.[19,21,22] HCT-15 cells (2×10^5 cells/well) were seeded in a 6-well culture plate and transfected with reporter plasmid (0.75 μg) and β-gal expression vector (0.25 μg) using Lipofectin® (GIBCO BRL, Grand Island, NY, U.S.A.). After transfection, cells were treated with 1 mM indomethacin with or without 1 μM RA pre-treatment. At the end of incubation, luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency by the corresponding β-gal activity. For statistical analysis, one-way analysis of variance was performed using GraphPad Instat® (GraphPad Software, San Diego, CA, U.S.A.). A value of $p<0.05$ was considered statistically significant.

DNA Fragmentation Assay DNA fragmentation was analyzed by agarose gel electrophoresis as described.[23] Briefly, HCT-15 cells were lysed in a buffer containing 20 mM EDTA, 0.05% (v/v) Triton X-100 and 10 mM Tris–HCl (pH 8.0). The fragmented DNA in supernatant was precipitated with ethanol and treated with RNase A and proteinase K. After electrophoresis on 1.8% agarose gels, the separated DNA was stained with ethidium bromide and visualized by UV light.

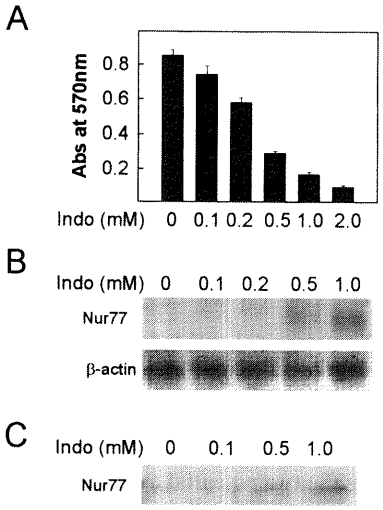

Fig. 1. Expression of Nur77 in Response to Indomethacin Treatment

The effect of indomethacin (Indo) on the viability of HCT-15 was measured by MTT assays (A). A total of 10000 cells per well was seeded in 96-well culture plates and treated with various concentrations of indomethacin for 12 h. Results represent mean±S.D. ($n=5$). The effects of indomethacin on the expression of Nur77 mRNA and protein were examined using Northern blot analysis (B) and immunoprecipitation/Western blot analysis (C), respectively. HCT-15 cells were incubated with the indicated concentration of indomethacin for 12 h.

RESULTS

Expression of Nur77 in Response to Indomethacin Treatment When HCT-15 cells were treated with indomethacin, the number of viable cells decreased dose-dependently; the decrease became statistically significant at 0.2 mM and reached about 90% at 1.0 mM (Fig. 1A). The expression of Nur77 mRNA and protein increased significantly following indomethacin treatment (Figs. 1B and 1C). The induction was clearly observed at 0.5 mM and was highest at 1 mM, which was consistent with the doses that induced an effective DNA fragmentation in a colon cancer cell line, HT-29.[7] Together, the results showed that indomethacin induced the expression of Nur77 at doses which caused significant cell death in HCT-15.

RA Repressed Induction of Nur77 Caused by Indomethacin Retinoids were shown to inhibit the activation-induced apoptosis of immature thymocytes and T-cell hybridomas.[24—27] We recently reported that RA repressed the induction of Nur77 that may cause the inhibition of activation-induced apoptosis of T-lymphocytes.[19] As shown in Fig. 2A, pre-treatment of RA also repressed the induction of Nur77 mRNA caused by indomethacin in colon cancer cells (Fig. 2A). The repression occurred in a time-dependent manner, in that it was observed as early as 2 h after RA pre-treatment. Next, we measured the amount of active Nur77 protein that bound DNA by gel-shift assays with an oligonucleotide probe containing the specific target sequences of Nur77, *i.e.*, NBRE.[28,29] When HCT-15 cells were treated with in-

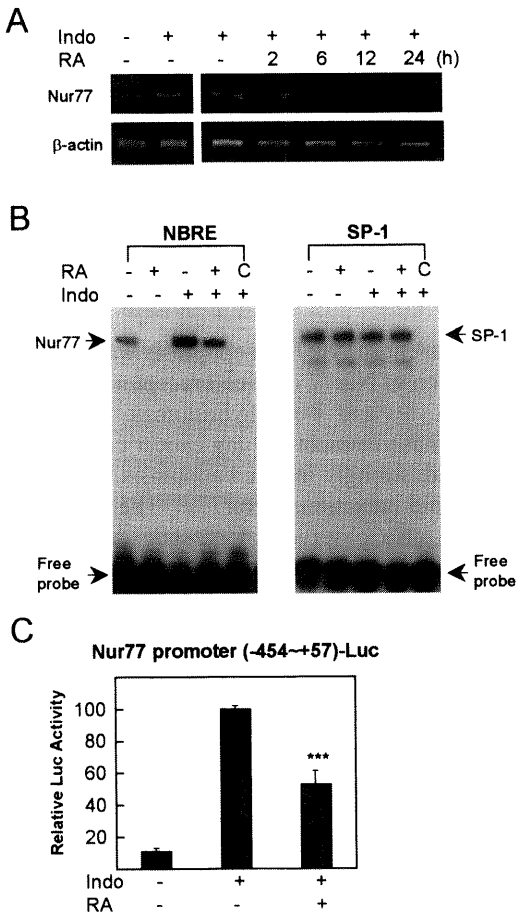

Fig. 2. RA Repressed Induction of Nur77 Caused by Indomethacin

(A) The effects of RA on the induction of Nur77 transcripts were examined using RT-PCR. HCT-15 cells were pre-incubated with 1 μM RA for the indicated time period, and then the cells were further incubated with 1 mM indomethacin (Indo) for 24 h. (B) HCT-15 cells were pre-incubated with 1 μM RA for 24 h, and then the cells were further incubated with 1 mM indomethacin for 24 h as indicated. Ten micrograms of nuclear extract was used for each reaction. The reaction mixtures were incubated with P^{32}-labeled NBRE as a probe and analyzed by gel shift assay. SP-1 binding was shown as control. C indicates a 100-fold excess amount of unlabeled NBRE used for competition to show specificity of the binding. (C) The Nur77 promoter (−454—+57)-Luc reporter, together with the β-gal expression vector, were transiently transfected into HCT-15 cells. Transfected cells were treated with or without 1 μM RA for 24 h and further incubated with 1 mM indomethacin for 12 h as indicated. After incubation, luciferase activity was measured and normalized by β-gal activity. Data represent mean±S.D. of 4 independent experiments. ***, $p<0.001$ *vs.* indomethacin-alone treatment.

domethacin, the amount of NBRE binding complex increased significantly (Fig. 2B). RA treatment inhibited significantly both the basal and indomethacin-induced DNA binding of Nur77.

The transcriptional activity of the Nur77 promoter has been demonstrated to play a crucial role in the high level of Nur77 protein expression during activation-induced apoptosis.[14,15] To confirm the transcriptional induction of Nur77 gene by indomethacin, we employed a Nur77 promoter-luciferase reporter containing sequences of −454 to +57 in the Nur77 gene promoter.[19] Transient transfection of this construct into HCT-15 showed about 10-fold activation when indomethacin was treated (Fig. 2C). When cells were pre-incubated with RA, the reporter gene activity was repressed approximately 50%. Taken together, our data clearly demonstrated that indomethacin induced Nur77 gene expression and that RA effectively inhibited this induction in HCT-15.

The AP-1-Like Motif in Nur77 Promoter Conferred Responsiveness to Indomethacin The Nur77 promoter from −454 to +57 contains several potential *cis*-acting regulatory elements, including binding sites for AP-1, RSRF and SP-1.[21] Therefore, the effects of indomethacin are possibly mediated through modulation of these transcriptional factors. As shown in Fig. 3A, indomethacin treatment significantly induced binding on AP-1-like sequences. An excess amount of unlabeled oligonucleotide-encoding consensus AP-1 and specific antibodies against c-fos and c-jun effectively abolished the binding, indicating that the binding complex consisted of AP-1 (Fig. 3A). RA significantly reduced both the basal and indomethacin-induced DNA binding on AP-1-like sequences (Fig. 3A). RSRF has been shown to be a major determinant responding to Ca^{2+} signaling in the course of Nur77 induction.[21] However, neither binding complexes nor changes with indomethacin treatment on RSRF binding sequences were observed in HCT-15 (data not shown).

Consistent with the DNA binding results, the AP-1-RE-luc reporter gene was activated by indomethacin and RA inhibited the activation of AP-1-RE-Luc (Fig. 3B). In contrast, RSRF-RE-Luc activity was unchanged by either indomethacin or RA treatment, whereas phorbol myristate acetate (PMA) (10 ng/ml)/Ionomycin (0.5 μM) induced the re-

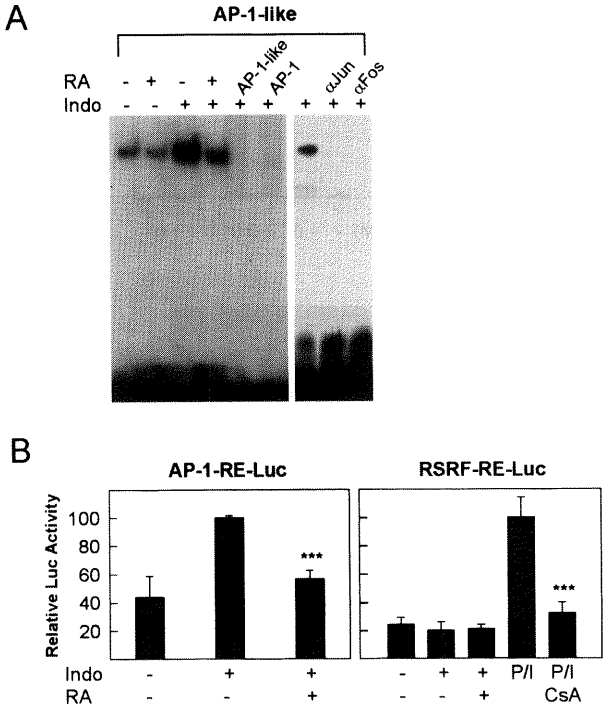

Fig. 3. The AP-1-Like Binding Motifs in Nur77 Promoter Conferred Responsiveness to Indomethacin

(A) HCT-15 cells were pre-incubated with 1 μM RA for 24 h, and then the cells were further incubated with 1 mM indomethacin (Indo) for 24 h as indicated. Ten micrograms of nuclear extract was used for each reaction. The reaction mixtures were incubated with P^{32}-labeled oligonucleotide containing AP-1-like sequences as probes and analyzed by gel shift assays. C indicates 100-fold excess amount of unlabeled probe used for competition to show specificity of the binding. Antibodies against Jun (αJun) and Fos (αFos) were used to show specific binding of AP-1. (B) Effects of RA on the transcriptional activities of AP-1-RE reporter gene. The indicated reporter gene together with β-gal expression vector was transiently transfected into HCT-15 cells. Transfected cells were pre-treated with or without 1 μM RA for 24 h and further incubated with 1 mM indomethacin for 12 h as indicated. PMA (10 ng/ml)/Ionomycin (0.5 μM) with or without Cyclosporin A (200 ng/ml) treatment was performed as positive control. After incubation, luciferase activity was measured and normalized by β-gal activity. Data represent mean±S.D. of 4 independent experiments. ***, $p<0.001$ *vs.* indomethacin-alone treatment.

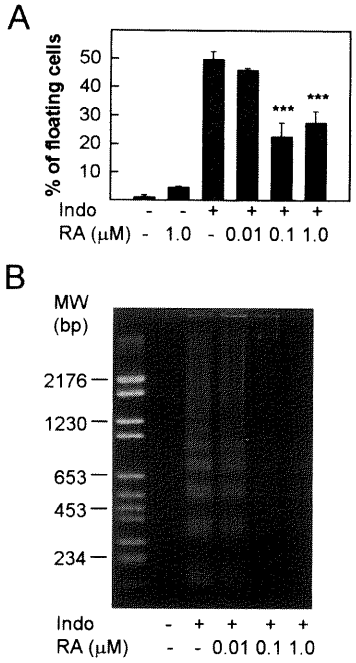

Fig. 4. Effects of RA on Apoptosis Induced by Indomethacin in HCT-15

(A) Measurement of floating cells: After incubation with the indicated concentrations of RA for 48 h, HCT-15 cells were further treated with 1 mM indomethacin (1 mM) for 3 h before floating and attached cells were counted. Data represent mean±S.D. of 3 independent experiments. ***, $p<0.001$ *vs.* indomethacin-alone treatment. (B) HCT-15 cells were treated with the indicated concentrations of RA for 48 h and then further incubated in the presence of 1 mM indomethacin for 3 h. Fragmented DNA was extracted, subjected to agarose gel electrophoresis, and stained with ethidium bromide.

porter activity which was repressed by Cyclosporin A (Fig. 3B). Taken together, our results suggested that indomethacin effectively induced Nur77 expression through activation of AP-1, but not by the activation of RSRF. Further, our results showed that the AP-1-mediated activation was sensitive to RA-induced antagonism.

RA Antagonized Apoptosis Induced by Indomethacin in HCT-15 Since RA repressed the transcriptional induction of Nur77, we speculated that RA might inhibit the indomethacin-induced apoptosis of HCT-15. When HCT-15 cells were treated with indomethacin, they became shrunken, detached and floated in the medium, which is a typical phenomenon observed in cells undergoing apoptosis (data not shown). As shown in Fig. 4A, approximately 50% of cells floated in the medium after indomethacin treatment. Interestingly, when cells were pre-treated with RA (0.1 and 1.0 μM), the number of these floating cells was reduced approximately 50%. Indomethacin caused DNA fragmentation in HCT-15 and it was inhibited by RA pre-treatment (Fig. 4B). In both cases, 0.1 μM all-*trans*-RA was as strong as 1.0 μM all-*trans*-RA in inhibiting DNA fragmentation induced by indomethacin (Fig. 4B). Taken together, our results indicated that a high level induction of Nur77 may have a role in indomethacin-induced apoptosis of colon cancer cells and that inhibition of the induction of Nur77 may be a mechanism inhibiting the apoptosis of these cells by RA.

DISCUSSION

A balance between cell production at the base and apoptotic cell death at the surface of the colonic crypts is precisely regulated to maintain cellular homeostasis in colonic epithelium. Therefore, progressive impairment of the apoptotic process in colonic epithelium may cause colon cancer.[30,31] In this regard, NSAIDs are of potential importance since they induce apoptosis of normal and neoplastic cells in the gastro-intestinal tract.[5,7,32,33] However, the molecular mechanism by which NSAIDs lead to the apoptotic process has not been clearly illustrated. In this report, we demonstrated that NSAIDs indomethacin induced the expression of Nur77 gene, which may result in apoptosis in colon cancer cells.

The involvement of Nur77 in apoptosis was first shown by a rapid induction of Nur77 on T-cell receptor signaling in immature thymocytes and T-cell hybridomas.[14,15] Recently, Li *et al.* showed that treatment of lung cancer cells with AHPN, a novel retinoid, strongly induced apoptosis that was accompanied by a rapid induction of Nur77.[16] The strong induction of Nur77 was also observed in prostate cancer cells after stimulation by a variety of apoptosis-inducing agents including etoposide,[17,18] suggesting that the transcriptional induction of Nur77 may be prerequisite to induce apoptotic cell death in a variety of cell-types following application of apoptotic stimuli. The mechanism by which Nur77 functions to induce apoptosis has not been clearly understood. Similar to other nuclear transcriptional factors, Nur77 possibly has a role in regulating the expression of modulators of apoptosis. Indeed, Weih *et al.* showed that fas Ligand (fasL) was upregulated in Nur77-transgenic thymocytes, suggesting that Nur77 may lead to apoptosis *via* the induction of fasL expression.[34] We have observed that the expression of a dominant-negative or an anti-sense Nur77 significantly blocked the induction of fasL in hepatoma cells (H.-J. Kang and M.-O. Lee, unpublished data), further supporting the role of Nur77 in fasL induction. Since the direct binding site for Nur77 was not found in the promoter of fasL, Nur77 may activate fasL by an indirect mechanism. Whether or not fasL is induced in indomethacin-treated HCT-15, and thereby results in apoptosis of the cells, remains as a subject for further study.

Retinoids, vitamin A and its natural and synthetic derivatives, regulate a wide array of biological processes including cellular proliferation, differentiation and development.[35] Retinoids are known to induce apoptosis in certain types of normal and cancer cells that may contribute to the known chemopreventive and chemotherapeutic effects of retinoids.[36,37] Confusingly, however, RA was also known to inhibit apoptosis of certain types of cells such as immature thymocytes, T-cell hybridomas and B-lymphocytes.[24—27] Essentially two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors were shown to be involved in this inhibition.[25—27,38] We have observed a significant induction of a subtype of retinoid receptor, RARβ, when HCT-15 cells were treated with RA.[23] Thus, RARβ may have a role in the repression of the Nur77 gene expression in HCT-15. Our data showed that the induction of Nur77 by indomethacin was mediated through the activation of AP-1 (Fig. 3), which could be well explained by the antagonism between retinoid receptors and AP-1.[39,40] RA inhibited the induction of Nur77, as well as the indomethacin-induced apoptosis of HCT-15, providing evidence that transcriptional induction of the Nur77 gene may be an important mechanism

by which indomethacin induces apoptosis in colon cancer cells (Figs. 1 and 4). Since retinoids are well known to exert chemopreventive and chemotherapeutic effects against a wide spectrum of cancers and have been used in the clinic for the treatment of human promyelocytic leukemia and breast cancer,[41,42] the pharmacological significance of the apoptosis-inhibiting effects of RA needs to be evaluated, especially when retinoids are administered in combination with other apoptosis-inducing chemotherapeutic drugs.

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