

Expansion of p75NTR/Oct4-Expressing Putative Stem Cells in HPV16-Transformed Precancerous Immortal Cell Lines under the Presence of TGF β and TNF α

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Abstract: We show here a possible *in vitro* model system for the analysis of relationship between our proposed p75NTR-expressing tissue-type-stem cell (TSC) candidate and HPV16-immortalized p75NTR-positive putative stem cell lines. We demonstrate that HPV16-transformed human cervical epithelial cell lines express Oct3/4 and contain p75NTR-positive cell subpopulation at various ratios in the immortalized cell population. We have isolated clonal variants (NCE16N+ and NCE16N-) that express p75NTR at high or low levels, respectively, and examined responses against inflammatory cytokines, TGF β and TNF α . We demonstrate that expression levels of p75NTR in the two distinct lines, NCE16N+ and NCE16N-, are correlated with differences in survival rates in the presence of TGF β . TGF β also induced epithelial-mesenchymal transition in these cell lines as demonstrated by the morphologic changes with expression of vimentin, enhanced migration, and induction of snail. TNF α alone had no effect on these processes, but was essential for apoptosis by co-stimulation with TGF β in NCE16N-. NCE16N+ was fairly resistant against apoptosis but sensitive to the inhibitory effect on the growth by these cytokines. Among receptors for these cytokines, the expression level of TGF β RII in NCE16N- was higher than that in NCE16N+. Hyper-phosphorylation of ERK1/2, p38MAPK and JNK occurred in NCE16N+ but not in NCE16N-. A specific inhibitor to p38MAPK influenced the survival rate in NCE16N-. These results implicate that the p75NTR-positive precancerous cell lineage will have an advantageous phenotype to survive longer under inflammatory situation.

INTRODUCTION

The etiology of cervical cancer has been linked to several types of human papillomaviruses (HPVs), known as a high-risk group for malignancy. Most typically, type 16 (HPV16) persists in lesions and may be critically involved in the progression from pre-cancerous cervical lesions towards malignancy [1, 2]. However, it usually takes a long time for the onset of malignancy. A number of previous studies have shown that this class of HPV can produce immortal cell line overcoming replicative aging *in vitro*, and thus the isolated precancerous cell lines have been expected to be a suitable model for carcinogenesis of human [3-7]. However, unlimitedly growing HPV-transformed human cells *in vitro* hardly develop towards malignancy. Exact mechanisms responsible for the long latency remain to be fully elucidated.

In this context, we here hypothesized the presence of adult tissue stem cells (TSCs) to be a target cell subset of HPV. In general, a close relationship between replicative aging of TSCs and cancer stem cells (CSCs) has been suspected, possibly because of the following aspects of these presumptive cell types [8-10]: (I) similarities in the mechanisms that regulate the self-renewal of TSCs and CSCs, (II) the possibility that most cancer cells that mature during a long latent period arise from a putative TSCs which retains

longevity, and (III) the notion that almost all malignant tumors should contain CSCs, a small and definable subset of cells with the ability to proliferate extensively and form tumors.

Insight into the origin of CSCs may shed light on the mechanisms of the multi-step carcinogenesis originating from TSCs [8-11]. Nevertheless, a possible linkage between TSCs and CSCs is not fully understood yet due to a lack of substantial knowledge on the cellular characteristics of TSCs and CSCs. A self-renewing and self-maintaining stem cell characteristics is crucial for not only homeostasis of various regenerative epithelia but also a genesis of evolving neoplastic cells. TSCs are thought to have self-renewing potential in response to tissue injury and other hyper-proliferative stimuli, such as inflammation [8, 9, 12, 13].

In an attempt to establish the cellular system for the analysis of HPV16-involved multi-step carcinogenesis, we have identified stem cell candidates as a possible target cell by HPVs [14-16]. This minor sub-population in normal epithelia co-expresses p75NTR, integrin β 4 and integrin β 1 [14, 15] and is mostly found in the basal layer of regenerative epithelia [14], where stem cells or progenitors have been postulated to reside [8-11, 14, 15]. Notably, a number of p75NTR-positive cells have been identified in developing cancer tissue [17]. The p75NTR-positive cells exhibited strong regenerative potential, unequal division, and long-term self-maintenance *in vitro* [15]. p75NTR has been

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known as a low-affinity neurotrophin receptor, a member of the TNF receptor superfamily [18].

In this study, we investigated a role of inflammation-associated cytokines in regenerative activities of the presumptive stem cells in target tissues of HPV16. We have focused on p75NTR as a stem cell marker to characterize the putative stem cells in normal ecto-cervical epithelial (NCE) cells and HPV16-immortalized cells (NCE16). We show here that the p75NTR-positive putative stem cell line (NCE16N+) has an advantageous potential to survive under the presence of TNF α and TGF β 1 *in vitro*.

RESULTS

HPV16-Transformed Human Epithelial Cell Lines Co-Expressing p75NTR, Integrin β 1, and Oct3/4

We have examined the expression level of stem cell-related marker such as Oct3/4, integrin β 1 and p75NTR by RT-PCR. The results demonstrated significantly high levels of Oct3/4 and integrin β 1 in all immortalized NCE cell lines and cancer derived cell lines (Fig. 1A). However, the expres-

sion level of p75NTR was various among the cell lines. Notably, HPV16-immortalized lines had stronger expression of p75NTR than cancer cell lines.

To examine if the event is specifically related with the function of HPV16, E6 and E7 were independently introduced into normal human cervical epithelial cells using the retroviral expression vectors (generously provided by Dr Kiyono, National Cancer Center). Notably, both E6 and/or E7 are responsible for the augmented expression of Oct 3/4 and Integrin β 1, and E6 alone had stronger effect on the expression of p75NTR. E7-transduced NCE cells (E7) exhibited relatively low expression of p75NTR (Fig. 1). The flow-cytometric analysis identified p75NTR-positive cells in the HPV16-immortalized lines (NCE16, NCE164 and NCE165) and confirmed that these immortal lines contained an increased number of p75NTR-expressing cells in comparison with that of untransformed NCE cells (Fig. 1B). Although the number of p75NTR-positive cells varied among the established immortal lines, these phenotypic traits were fairly stable under our consistent culture conditions.

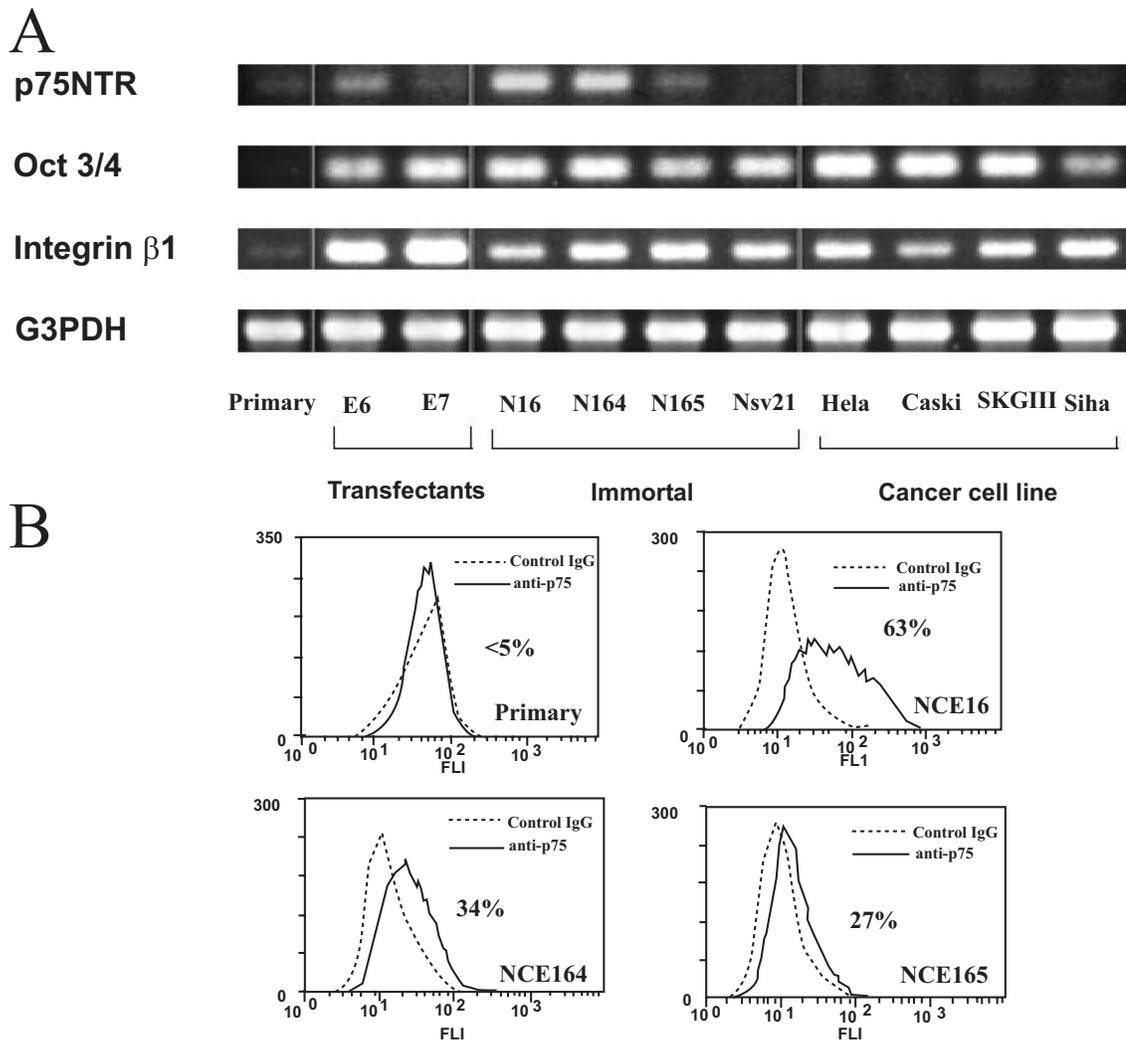


Fig. (1). **A.** RT-PCR detected p75NTR, Oct3/4, and Integrin β 1 in various cell types: the primary NCE, E6, E7, NCE16, NCE164, NCE165, and Nsv21. Nsv21 is a SV40-transformed immortalized line. **B.** Increased numbers of p75NTR-positive cells in the independently isolated HPV16-immortalized cell lines (NCE16, NCE164, and NCE165) in comparison with the primary cultured normal cervical epithelia cells as demonstrated by flowcytometry.

Two Distinctive Clonal Variants in Terms of the Expression of p75NTR

Although p75NTR-positive cells were predominant (63%) in NCE16 line, the spectrum of the expression levels of an independent each cell in the population was broad. In an attempt to characterize the phenotypic difference associated with the expression level of p75NTR in HPV16-transformed cells, we isolated two-clonal variants. One of which, namely NCE16N+, is exclusively constituted of p75NTR expressing subsets and the expression level of p75NTR was significantly higher than that of NCE16N-, the lower expressing clonally derived variant (Fig. 2A). The expression levels of involucrin confirmed that the cells in NCE16N+ variant mostly represented an undifferentiated phenotype and the other NCE16N- variant contained some differentiating subpopulation at the same levels as the parental NCE16 (Fig. 2B).

Epithelial Mesenchymal Transition (EMT) and Apoptosis by the Effect of TGF β and TNF α

To examine the fate of the p75NTR-expressing cell subset in NCE16 cells, NCE16N+ and NCE16N- cells were exposed to TNF α and/or TGF β . TNF α alone had no visible effect on cellular morphology. In contrast, TGF β caused a flattened cytoplasmic extension in both cell lines as noted under the phase contrast microscope (Fig. 3A and B, upper panels). Immunological examination using a specific antibody demonstrated that the morphologic change is associated with the expression of vimentin, suggesting that TGF β affects EMT in both lines. Moreover, we found that co-treatment with TNF α and TGF β induced a frequent cell death in the NCE16N- (Fig. 3A and C, N-).

In contrast, the NCE16N+ line was fairly resistant to death by many cytokines and growth factors tested and combinations thereof (Fig. 3B and C, N+, and data not shown). However, untransformed primary NCE cells and a parental

line, NCE16 also contained some apoptotic figures in response to the cytokines (Fig. 3C).

TGF β Responsible for Augmented Cell Migration but not Directly for Cell Death by TNF α

TGF β induced the most significant phenotypic changes, an epithelial mesenchymal transition (EMT) in NCE16 as suggested by the induced vimentin expression (see Fig. 3). This confirms the enhanced cellular motility and expression of other EMT-associated genes such as those for snail and N-cadherin in the presence of TGF β . The result shows that the enhanced cell migration was specifically associated with the events induced by TGF β alone but not with TNF α . Although TNF α alone had little effect on motility in the NCE16N+ or NCE16N- line, it appeared to suppress the enhanced motility induced by TGF β (Fig. 4A).

RT-PCR revealed that there are some differences in the expression of snail and N-cadherin between the two lines, NCE16N+ and NCE16N- (Fig. 4B). The expression of snail was up-regulated within 24 h in both lines under the presence of TGF β 1. This indicates that TGF β -signaling is involved in the snail induction with time in both lines. Co-treatment with TNF α and TGF β for longer than 24 h down-regulated the expression of snail at mRNA level to some extent in both lines. The enhanced expression of N-cadherin by these cytokines was also detected at the same levels in both cell lines (Fig. 4B). Although TGF β was necessary for inducing EMT in HPV16-transformed cells, these results suggest that crosstalk between TGF β and TNF α could be a key for death or survival of HPV16-immortalized precancerous cell lines.

Restoration of NCE16N+ Cells after a Long-Term Survival in the Presence of TNF α and TGF β

To further investigate the differential sensitivity of N+ and N- cells to the cytokines, the cells were exposed to vari

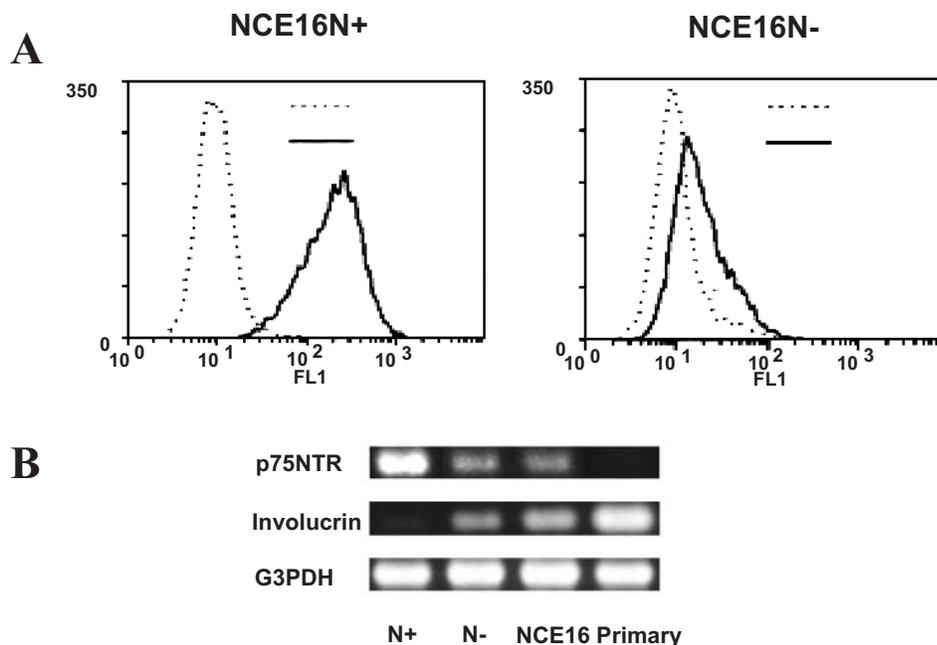


Fig. (2). A. Flowcytometry of two clonal variants expressing p75NTR at high levels (NCE16N+) and low levels (NCE16N-) B. Involucrin as a marker for the differentiation was detected by RT-PCR. N+: NCE16+, N-: NCE16N-.

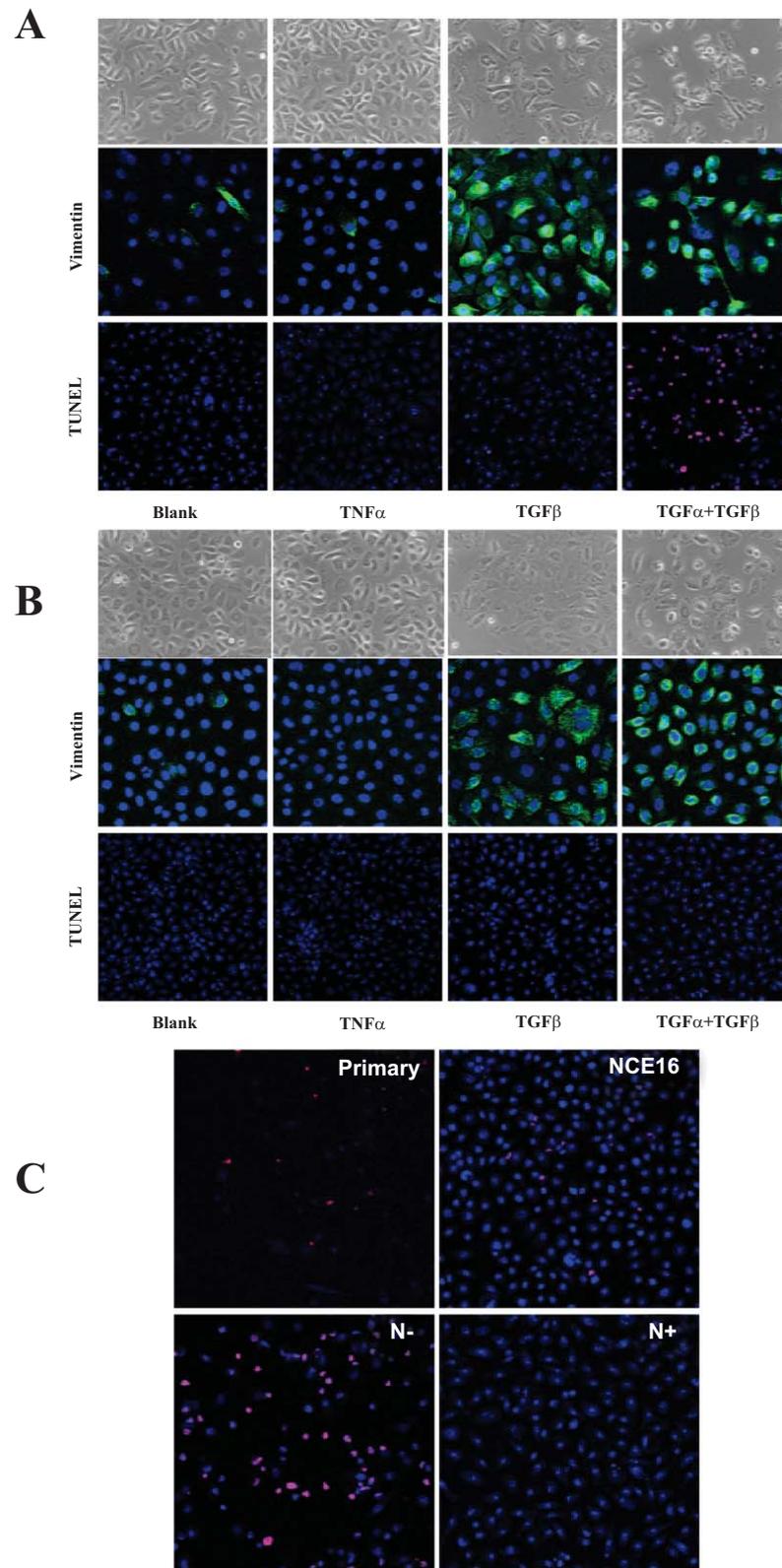


Fig. (3). EMT and apoptosis by the effect of TGF β and TNF α . NCE16N+ and NCE16N- cells were cultured in the absence (Blank) or presence of TNF α (10 ng/ml), TGF β (10ng/ml), or both cytokines (TNF α + TGF β) for five days. Vimentin expression and TUNEL-signal were detected using a fluorescence confocal microscope. Bright fields (**A** and **B**, upper panels), Vimentin expression (**A** and **B**, middle panels) and TUNEL assay (**A** and **B**, lower panels) in NCE16N- (**A**) and NCE16N+ (**B**). **C** shows a magnified view of a typical apoptotic figure shown in **Panels A** and **B**. N+: NCE16N+, N-: NCE16N-. Primary: Untransformed NCE cells. NCE16: parental HPV16-jmmortalized line.

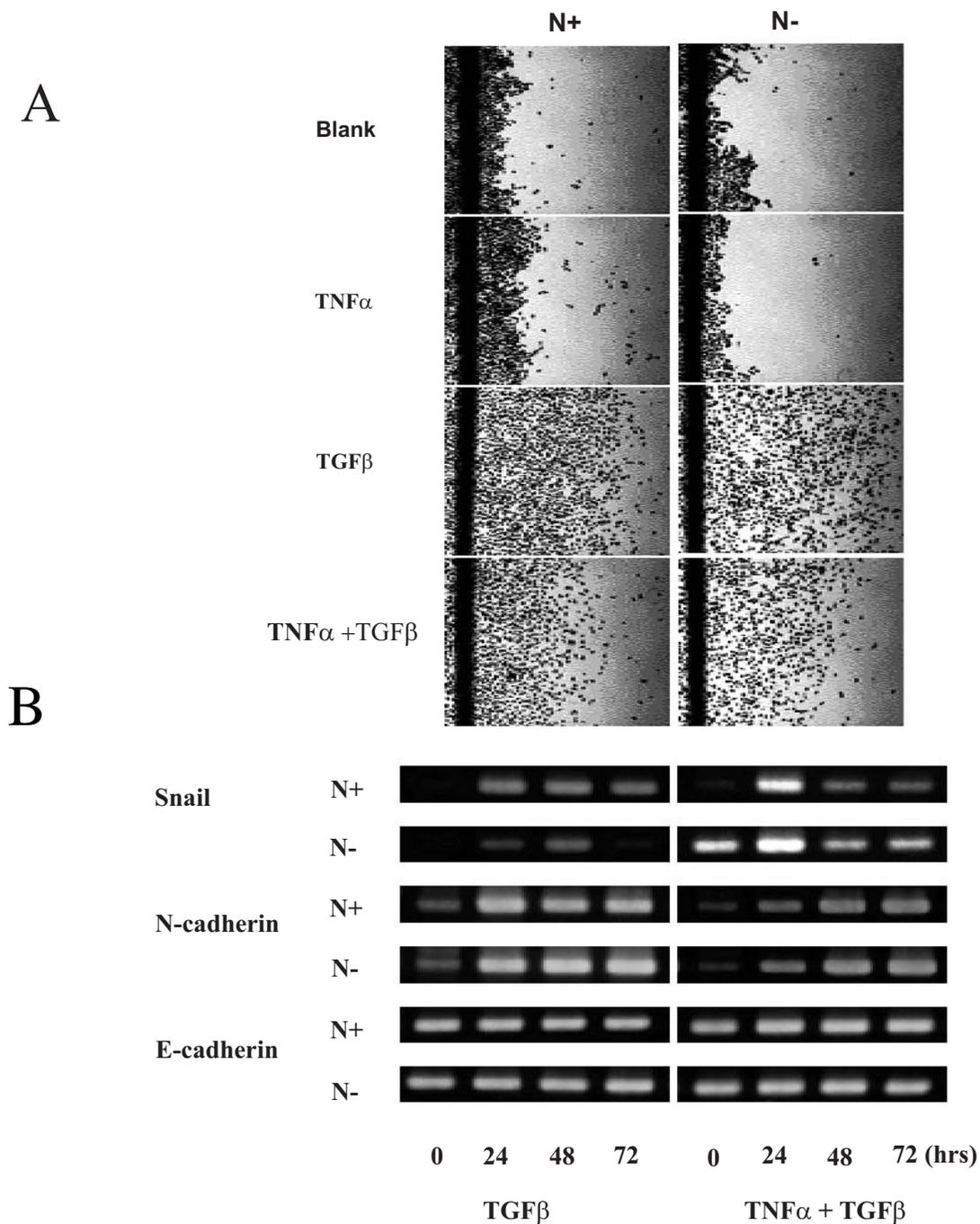


Fig. (4). **A.** Epithelial-mesenchymal transition (EMT) in NCE16N+ and NCE16N- cells in the presence of TGF β or both TNF α and TGF β . Motility of N+: NCE16N+ cells, and N-: NCE16N- cells, after culturing with no cytokines (Blank), TNF α , TGF β , or both cytokines (TNF α +TGF β). After culturing with cytokines for four days, the cells were plated on another culture dish that was partially covered with a silicon sheet, and cultured overnight until cell adherence. Then the silicon sheet was removed from the dishes. Cell motility was observed under a phase-contrast microscope on two days after the sheets were removed. Original magnification: 10 x 5. **B.** Gene expression of snail, N-cadherin, or E-cadherin in NCE16N+ (N+) and NCE16N- (N-) at 0, 24, 48 or 72 hours in the presence of TGF β or both TNF α and TGF β (each concentration 10 ng/ml). Snail, N-cadherin, and E-cadherin are an EMT-responsible gene, a mesenchymal marker, and an epithelial marker, respectively. RT-PCR was carried using a specific pair of primers as shown in Table 1.

ous concentration of TNF α in the presence of TGF β for 5 days (Fig. 5A). The results demonstrated that N- cells were very sensitive to the TGF β and only less than 40 percent of the cells survived, mainly due to apoptosis and growth suppression in part (see Fig 3C). In contrast, N+ cells appeared

to be fairly resistant to TGF β and approximately 85 percent of the cell could survive. However, growth suppression and/or cell death in both lines, N+ and N-, were significantly induced by high concentrations of TNF α in a dose response manner.

Following the loss of most viable N⁺ cells, the resistant cells against the cytokines in the fraction of NCE16N⁺ start to grow as a clonal variant and expanded to subsequently become predominant (Fig. 5B). To monitor the population dynamics of the p75NTR-positive cell subset, flowcytometry was performed for NCE16N⁺ cells co-treated with TNF α and TGF β (Fig. 5C b, c and d). The results demonstrated that the number of p75NTR-positive cells remarkably decreased within two weeks. The remaining cells in the population at this stage were p75NTR-dim cells (Fig. 5C b), mostly differentiated (involucrin-positive, data not shown). Following this stage, the number of p75NTR-bright cells increased concomitantly with the appearance of expanding colonies by the 4th week (Fig. 5C c). Almost all cells were p75NTR-high cells in the 6th week (Fig. 5C d). The expression levels of the receptors for TNF α and TGF β were examined by RT-PCR and detected relatively lower levels of the type II TGF β receptor (TGFRII) in NCE16N⁺ at week 6th after the co-treatment with TNF α and TGF β (Fig. 5D).

The Fate-Determination Signaling via TNF α and TGF β in NCE16N⁺ and NCE16N⁻ Cells

To focus on a possible signaling pathway of TNF α and TGF β in NCE16N⁺ and NCE16N⁻ cells, RT-PCR was carried out to determine levels of the receptors for TNF α and TGF β , including type I (TNFRI) and type II (TNFRII) TNF receptors and type I (TGFRI) and type II (TGFRII) TGF β receptors. The NCE16N⁺ line had lower levels of TGFRII than the NCE16N⁻ line. There was little difference in the expression of the other receptors (Fig. 6). These results also suggest that the effect of TGF β 1 via the TGFRII receptor predominates in apoptotic cell death and/or growth suppression in NCE16N⁻ cells in the presence of TNF α .

We next examined mitogen-activated protein kinase (MAPK) pathways as a possible signaling for apoptosis in NCE16N⁺ and NCE16N⁻, since MAPK signaling is known to involve in apoptosis induced by either TNF α or TGF β . The results in Figure 7 show that hyper-phosphorylation of p38MAPK, JNK, and Erk1/2 occurred in NCE16N⁻ (Fig. 7A, D and G). Chemical inhibitors, SB203580 and U0126A, specific for the phosphorylation of p38MAPK and MEK1/2, respectively, suppressed reduction of cell number with a statistical significance in NCE16N⁻ under the presence of TNF α and TGF β (Fig. 7B and H). However, there is little statistical significance in the inhibitory effect of SB203580 on the NCE16N⁺ (Fig. 7C), strongly suggesting that p38MAPK pathway effectively works as apoptotic signaling only in NCE16N⁻. On the other hand, MEK1/2 inhibitor U0126A blocked reductions of cell number to some extents in NCE16N⁺ (Fig. 7I). Since the decreases of number of cells by the higher doses of TNF α under the presence of TGF β could be the suppression of cell growth but not due to apoptotic event in NCE16N⁺ (see Fig. 5A), Erk pathway might work as cell growth signaling with little effect on apoptosis. While, the JNK inhibitor was not effective on the suppression of cell death or cell growth in both NCE16N⁻ and NCE16N⁺ (Fig. 7E and F).

DISCUSSION

The p75NTR-expressing cells have been suspected to be presumptive TSCs residing in various regenerative adult human epithelia [14,15] such as the skin, esophagus, oral

cavity, and uterine cervix. Malignantly transformed squamous cell carcinoma and their cell lines also contain a p75NTR-expressing subset [17, and our unpublished observations] This particular cell type represents slow cycling or resting *in vivo* as well as *in vitro* and self-renewing potential *in vitro* [14,15]. Longevity is another crucial characteristic of TSCs, but they undergo replicative senescence under normal circumstances, even though telomerase activity is detected [10, 14, 16, and our unpublished results]. These characteristics are particularly important for the genesis and development of cancers in terms of CSCs. We have shown here that HPV16-involved immortalization of human regenerative epithelial cells could serve as a model system for analyzing the close relationship between TSCs and CSCs. Since the immortalized cell lines used here were independently established in a separate experiment after transfecting HPV16 DNA into cells derived from different individuals over the past 20 yrs, the present results strongly suggest that HPV16-involved immortalization of human cervical epithelial cells preferentially occurred among subsets of TSCs or progenitors. In addition to the analyses of HPV16-immortalized cell lines, the transformation of E6 and E7 of HPV16 using retroviral vectors more or less augmented the expression of p75NTR (see Fig. 1B). Some variations in the expression level of p75NTR among cells and cell lines, may be reflected by the differentiated state of the transformants (data not shown). These results implicate that selective stimulation to proliferate by the virus oncogenes may occur in stem cells or progenitors upon HPV16-infection in cultured cervical epithelial cells. Moreover, the expression of Oct3/4 and integrin β 1 augmented in this process.

TNF α and TGF β play critical roles in inflammation and tissue repair. TNF α is a pro-inflammatory cytokine, which produces other inflammatory cytokines and controls the inflammatory cell population [19]. TGF β interferes this process by suppressing immunological responses and accelerates tissue repair by inducing production of the extra-cellular matrix and epithelial differentiation [20]. A chronic inflammation seems to be due to persistence of the initiating factors or a failure of mechanisms required for resolving the inflammatory responses [21]. In this study, we have shown that the effect of TNF α and TGF β on two distinctive immortalized cell lines, in which p75NTR was differentially expressed, may a suitable *in vitro* model for chronic inflammation. As shown in Fig. 3C, NCE16N⁻ cells underwent apoptosis, though NCE16N⁺ cells did not, under the presence of both TNF α and TGF β . In addition, a subpopulation of surviving NCE16N⁺ cells proliferated with strong expression of p75NTR after long-term co-treatment with TNF α and TGF β (also see Fig. 5D). The p75NTR-negative cell dominated in the normal epithelium with the differentiated phenotype. These results suggest that chronic inflammation injures a major part of various epithelia consisting of the p75NTR negative cells. Stem cells and/or progenitors might have accumulated at lesions when chronic inflammation occurred in HPV16-transformed cells.

Previous studies have shown that EMT characterizes the progression of many carcinomas and is linked to deteriorated cell-cell adhesion and that cell motility relied on invasion and metastasis [22,23]. The present observations demonstrate that TGF β -mediated signaling plays a critical part in EMT in association with apoptosis and cell migration linked

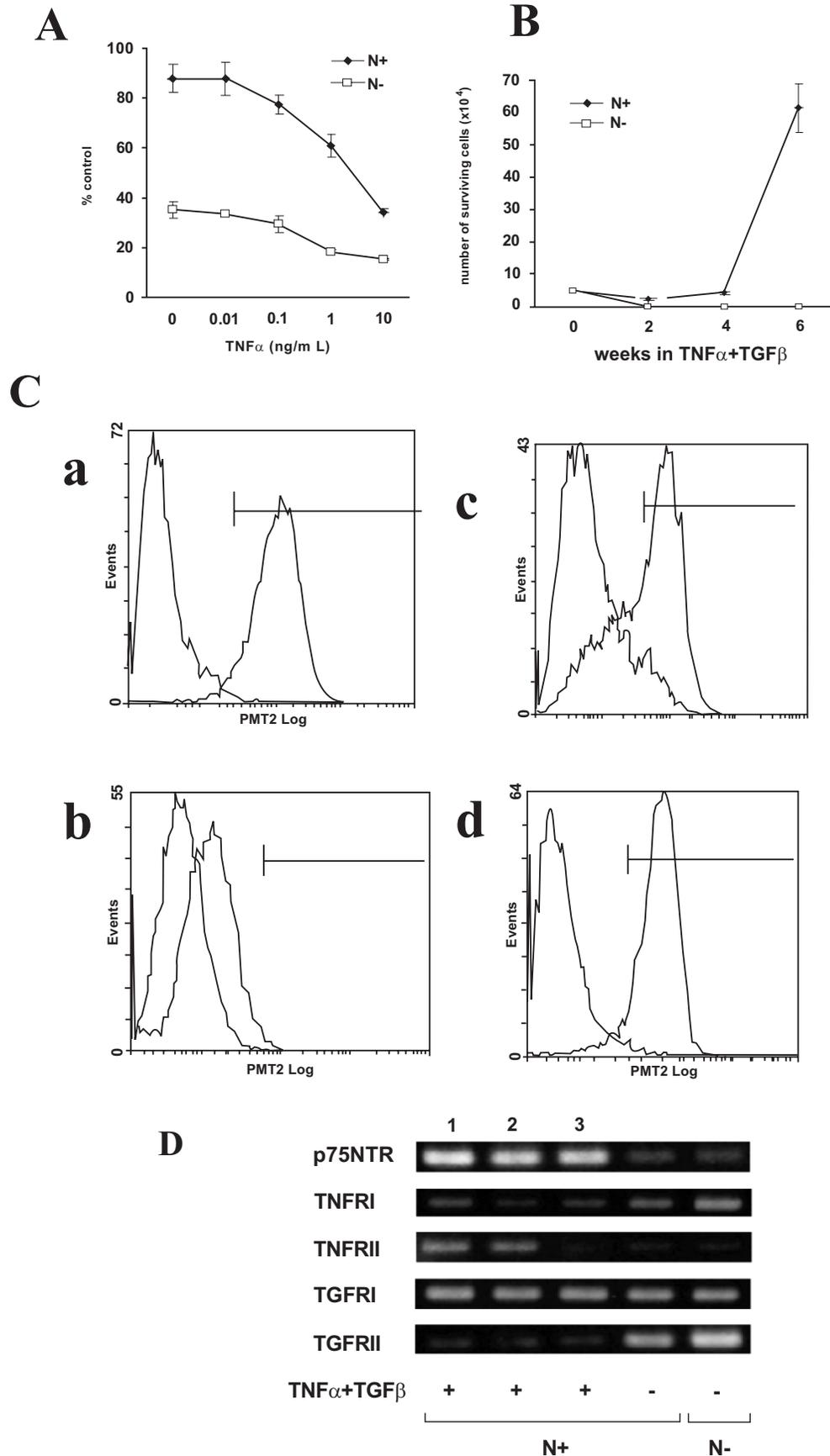


Fig. (5). **A.** Survival rates of N+ (NCE16N+) and N- (NCE16N-) in the presence of TGFβ and various concentrations (0, 0.01, 0.1, 1.0 and 10 ng/ml) of TNFα for 5 days. **B.** The fate of N+ and N- after long-term cultivation in the presence of TNFα (10ng/ml) and TGFβ (10ng/ml). The

(Legend Fig. 5) contd....

number of NCE16N+ (N+) and NCE16N- (N-) cells in zero, two, four and six weeks after culturing with both TNF α (10ng/ml) and TGF β (10ng/ml). The surviving cells were counted with the trypan blue dye exclusion method. Each dot with a bar represents the mean and S.D. of a triplet culture C. Flowcytometric analysis of population dynamics of p75NTR-positive and negative cells in the NCE16N+ during the treatment with both TNF α (10ng/ml) and TGF β (10ng/ml). a. Untreated NCE16N+. b. 2 weeks. c. 4 weeks. d. 6 weeks. p75NTR-positive fractions were indicated by bars of solid lines in the chart. Far left peaks in the chart indicate the profile with IgG. D. RT-PCR for the expression of p75NTR, type I or type II TNF α receptors (TNFRI or TNFRII), and type I or type II TGF β receptors (TGFRI or TGFRII). 1, 2 and 3 indicate triplet samples of survivors after culturing six weeks with TNF α and TGF β . RT-PCR was carried out using the primer set listed in Table 1.

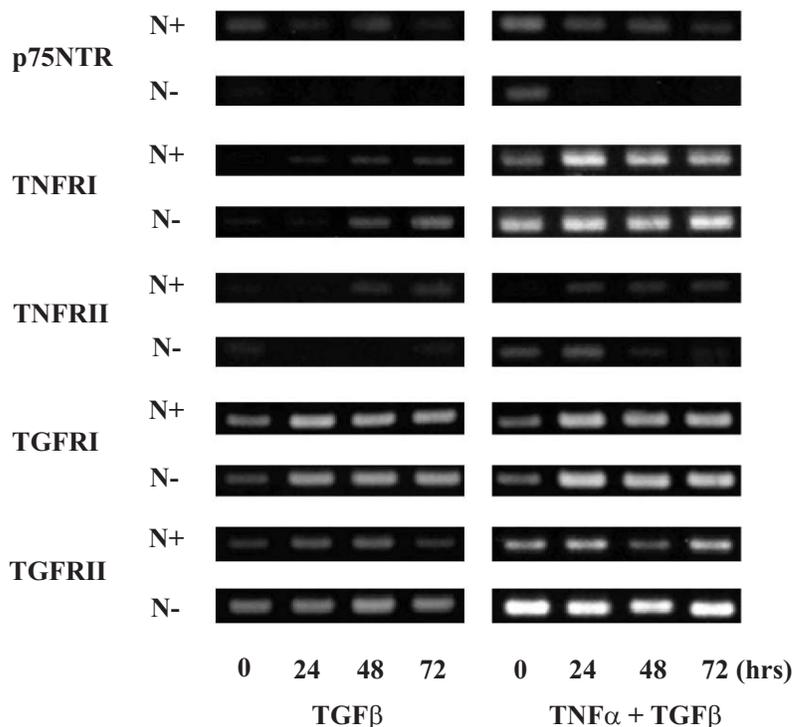


Fig. (6). Expression levels of receptors for TNF and TGF β in the NCE16 lines (N+ and N-), respectively. Gene expression of type I or type II TNF α receptors (TNF-RI or TNF-RII), and type I or type II TGF β receptors (TGF-RI or TGF-RII) in NCE16N+ (N+) and NCE16N- (N-) 0, 24, 48 or 72 hours after culturing with TGF β or with both TNF α and TGF β with a specific pair of primers as shown in Table 1.

to signaling of TNF α . NCE16N+ showed lower expression levels of TGFRII than NCE16N- (see Fig. 6). TGF β -mediated signaling is necessary for TNF α -directed apoptosis of parental NCE16 cells. These results imply that survival or apoptosis is determined through TGF β /TGFRII rather than TGFRI. As for downstream of TNF α and TGF β , we identified the activation of p38 MAPK, which should be linked to apoptosis as shown in various cell lines such as prostate cancer, murine myeloid cells, and human hepatoma [24,25]. Our present study demonstrated that the phosphorylation of p38MAPK seems critical in determining the fate of the given cell type in which p75NTR is differentially expressed as shown in our distinctive clonal cell lines, NCE16N+ and NCE16N-. These results further strengthen the notion that inactivation of the TGF β signaling pathway is implicated in various human malignancies [26-29]. Moreover, our data suggest that TGF β acts on most normal epithelial cells in cooperation with TNF α as a death factor, resulting in the accumulation of p75NTR-positive cells. Although the exact mechanisms for the selective expansion of TSCs or a TSC lineage that predispose to cancer are largely remained to be clarified, the results obtained with our *in vitro* model suggest

factors that may be involved in apoptosis, differentiation, and survival. These cellular events are closely related to mechanisms for the fate determination of TSCs or predisposition to CSCs.

In conclusion, HPV16-oncogenes facilitate the survival of the fittest in the immortalized p75NTR-positive epithelial stem cells under the presence of TNF α and TGF β , implicating that HPV16-infected human cervical TSC may be expanded in the early phase of the virus-involved progressive transformation.

MATERIALS AND METHODS

Normal Cervical Epithelial Cell

Normal cervical epithelial (NCE) cells were prepared from uterine cervixes surgically removed from patients with severe uterine myoma as described previously [12-14]. Briefly, cervical tissue specimens were incubated in 1000 units of dispase (GIBCO-BRL, Inc.) at 4°C, overnight. The detached cervical epithelium was minced into small pieces and incubated in Ca²⁺, Mg²⁺ free PBS (PBS(-)) containing 0.01% EDTA solution at 4°C for 20 min then pipetted sev-

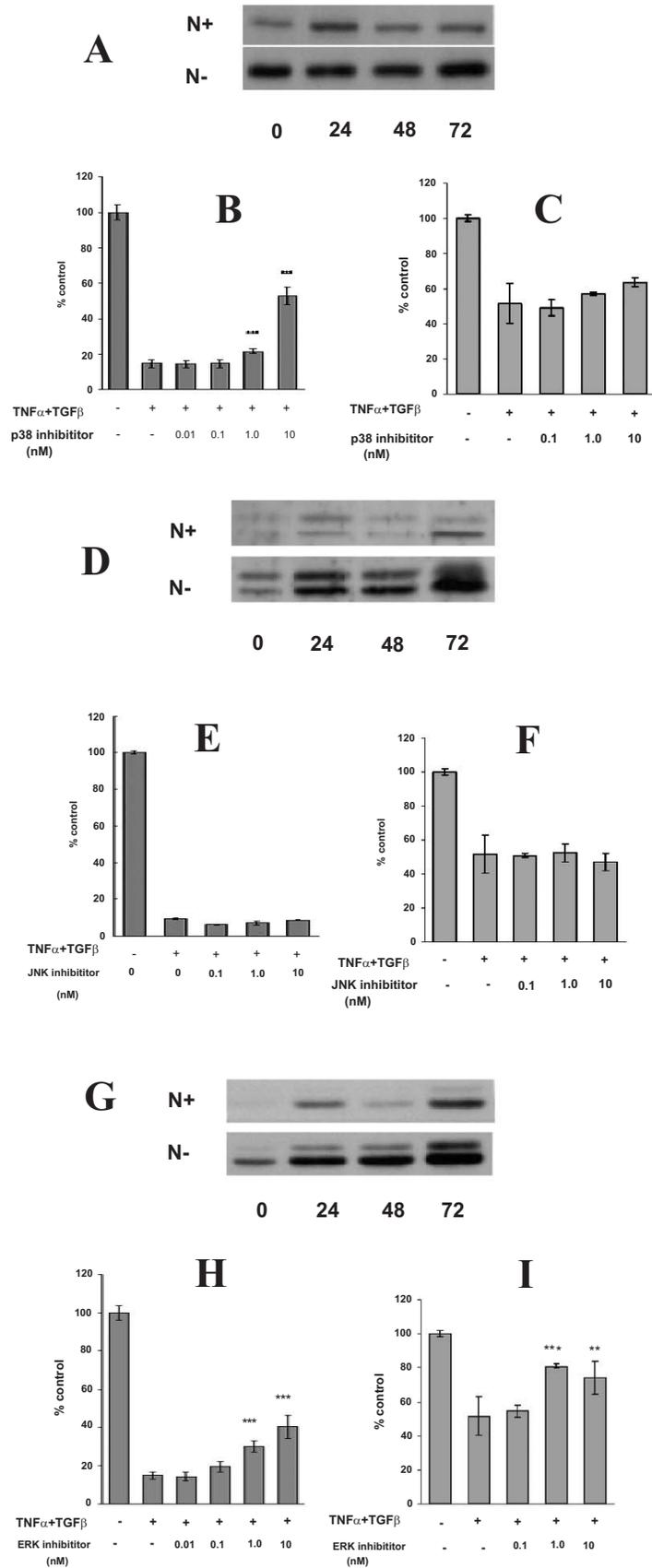


Fig. (7). A possible key enzyme for growth suppression and/or cell death induced by co-treatment with TGF β and TNF α in NCE16N-. Western blotting using specific antibody detected phosphorylation of p38 MAPK (A), JNK (D) and Erk1/2 (G) in NCE16N+ (N+) and NCE16N-

(Legend Fig. 7) contd....

cells (N-) 0, 24, 48 or 72 hours after culturing with both TNF α and TGF β . Suppressive effects on cell death in NCE16N- (B, E, H) and NCE16N+ (C, F, I) by specific kinase inhibitors of p38 MAPK (B and C), JNK (E and F) and Erk1/2 (H and I) in the presence of TNF α and TGF β . Cell viability was measured by the-MTT method, and is represented as a percentage of the control value (without treatment of cytokines and the chemical inhibitor). Each column with a bar represents the mean and S.D. of triplicate cultures. **, p<0.01, ***, p<0.001 (vs. control, the Dunnett test).

eral times. The dissociated single cells were collected through 80 platinum mesh. NCE cells were separately cultured in MCDB153 medium containing a low Ca²⁺ concentration (0.03 mM) supplemented with hormone mixture and bovine pituitary extract (75 μ g/ml) (03 complete medium). These cells were serially cultivated by 1:5 splits for at least five passages in a humidified 5% CO₂ atmosphere at 37°C as described.

Transfectants and Cell Lines

E6 and E7 transfectants were obtained by transfection of NCE cells (5.0×10^5) with the retroviral vectors pCLXSN-16E6SD and pCLXSN-16E7, respectively. NCE16, NCE164 and NCE165 cells were established as immortalized-lines of NCE cells after transfection of HPV16 DNA [12, 13, this study]. NCE16N+ and NCE16N- cells were clonal variants isolated from the parental NCE16 cell line after fractionation with Dynabeads using anti-p75NTR antibody (Dako Cytomation, Glostrup, Denmark). NCE16 cells were washed three times with cold PBS(-) and resuspended in an appropriate volume of PBS(-) at 1.0×10^7 cells/ml. Anti-p75NTR antibody was added at a concentration of 250 ng/ml, and then incubated at 4°C for 30 min with constant agitation. The cells were washed with PBS(-) three times and resuspended into new tubes at a concentration of 1.0×10^7 /ml with PBS(-). IgG-conjugated magnetic particles (Dynabeads M-450, DYNA-LAS Co., Norway) were added at a ratio of four particles/ cell, incubated gently with agitation at 4°C for 30 min. Cells bound to Dynabeads were separated under magnetic fields using Dynal MPC[®] -M for 1 min. Unbound cells were

recovered and washed three times with PBS (-). The bound or unbound cells were cultured after dilution (single cell/well) in 96-well culture plates. The clonal cells with the highest and lowest-levels of p75NTR were named NCE16N+ and NCE16N-, respectively. These NCE lines were cultured with 03 complete medium.

Polymerase Chain Reaction (PCR)

Total RNA was purified from cells using Tetrasol[®] (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized from 1 μ g of total RNA using SuperScript III reverse transcriptase (Invitrogen Inc., Carlsbad, CA). The cDNA samples were amplified in a DNA thermal cycler (PE Applied Biosystems, Foster City, CA) for 30 to 35 cycles (30-s denaturation at 94°C, 1-min annealing at 53°C to 55°C, and 1-min elongation at 72°C) with Ex Taq enzyme (Takara Inc., Kyoto, Japan). The primer sequences are listed in Table 1.

Flowcytometry

Trypsinized cells were washed once in cold PBS(-), and 2×10^5 viable cells were resuspended in 50 μ l of PBS(-) with 0.5% BSA (staining buffer). Cells were incubated with 1 μ g/ml of mouse anti-p75NTR (clone NGFR5, Dako) for 30min at 4°C. Nonspecific isotype-matched antibodies were used as controls. After being washed with staining buffer, cells were resuspended with 50 μ l of staining buffer and incubated with 1 μ g/ml of FITC-conjugated goat anti-mouse IgG for 30min at 4°C. After further washing with staining buffer, cells were resuspended with 5 μ g/ml of potassium

Table 1.

Target Gene	Forward Sequence	Reverse Sequence
p75 ^{NTR}	TGAGTGCTGCAAAGCCTGCAA	TCTCATGCTGGTAGTAGCCGT
Oct4	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGGGTTTCGGGCA
Integrin betal	GTGTGGCCCAAGACAGTTCT	GGTTACCCACCCTCTGACT
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
Involucrin	TGTTCTCCAGTCAATACCC	ATTCTCATGCTGTTCCAGTGC
Shal	AATCGGAAGCCTAAATACAGC	AGGAAGAGACTGAAGTAGAG
E-cadherin	TCTACAGCATCATGCCAAGGAGCTG	AGCTTGAACCAGGTATACGTAGG
N-cadherin	TGCGGTACAGTGTAAGTGGGCCAGG	CGATCAAGTCCAGCTGCCACTGIG
TGFR1	AGATTACCAACTGCCTTAT	ATTCTTCTGTTCCCTCTCA
TGFR2	GCAGTGGGAGAAGTAAAAG	GTTTAGGGAGCCGTCTICA
TNFR1	GCTCCTGGAGCTGGTGGGAA	CAGGAGAGGTGCACGGTCCCATT
TNFR2	CTCAGAGAATACTATGACCAG	CCTGCAAATATCCGTGGATG

iodide (PI)-PBS(-), and analyzed by Epics Altra (Beckman Coulter Inc., Fullerton, CA) using EXPO32 software (Beckman Coulter Inc.). Dead cells positive for high PI staining were eliminated.

Fluorescence Immunocytostaining

Cells were cultured with 10 ng/ml of TNF α and/or TGF β (PeproTech, Rocky Hill, NJ) for 5 days. The cells were fixed in 4% paraformaldehyde in PBS(-), and washed with PBS(-) three times. The cells were blocked with staining buffer, and incubated with mouse anti-vimentin antibody for 60min at room temperature (RT). Nonspecific isotype-matched antibodies were used as controls. After being washed with staining buffer, cells were incubated with FITC-conjugated goat anti-mouse IgG for 60min at RT. After further washing with staining buffer, images were captured using a confocal laser scanning microscope (Axiovert 200 M, Carl Zeiss Inc., Göttingen, Germany), and analyzed with PASCAL software (Carl Zeiss Inc.).

Analysis of Apoptosis.

Cells were cultured with 10ng/ml of TNF α and/or TGF β for 5 days. Apoptosis was detected by the TUNEL method with an *in Situ* Cell Death Detection Kit, the TMR red kit (Roche Applied Science, Penzberg, Germany). Images were captured using the Axiovert 200 M, and analyzed with PASCAL software.

Western Blotting

Cells harvested at a concentration of 5 x 10⁶/ml were treated with lysis buffer (10mM Tris-HCl, 1% SDS, pH 8.8). The protein concentration in the centrifuged extracts was estimated using the Bio-Rad Protein assay (Bio-Rad Laboratories Inc., Hercules, CA). After boiling for 2min, lanes were loaded with extracts, using a total protein concentration of 1.0 μ g for the detection of phosphorylated JNK, p38MAPK or Erk1/2. After electrophoresis through 11% polyacrilamide gels, proteins were transferred electrophoretically to PVDF membranes (Millipore Corp., Bedford, MA). Membranes were blocked with 2% skim milk in 0.1% Tween-20 in TBS before incubation with primary antibody. The phosphorylated proteins were detected with a MAPK family protein kit (Signal transduction Lab., Beverly, MA) and enhanced-chemiluminescence (ECL) reagents (Amersham Bioscience Corp., Piscataway, NJ) according to the manufacturer's instructions. Finally, the proteins were visualized on X-ray film.

Cell Viability

Cells (5 x 10³ /well) were cultured with TNF α and/or TGF β on 96-well culture plates for 5-7 days. In experiments on the involvement of the MAPK pathway in apoptosis, SB203580, U0126A (Signal transduction Lab.), and JNK inhibitor II (Calbiochem Co., La Jolla, CA) were used as inhibitors of the phosphorylation of p38MAPK, Erk1/2, and Jun kinase, respectively. Cells were treated with cytokines and MAPK inhibitors in the same time period. Cell viability was estimated using TetraColor ONE (Seikagaku Co., Tokyo, Japan), and A450 was measured with a plate reader (AURO_{MX}/Light, Perkin Elmer Japan, Kanagawa, Japan). For long-term treatment with cytokines, 5 x 10⁴ cells were

cultured in 60-mm culture dishes. Cells were diluted before becoming confluent. Cell viability was estimated with the trypan blue exclusion method.

Cell Motility Assay

Silicon rubber sheets were cut to an adoptive size, and plated on 60mm culture dishes after being autoclaved. After a pre-treatment with the cytokines for 5 days, cells were cultured on these dishes overnight, and then the silicon rubber sheets were removed. Cell motility was observed under the microscope after culture for 2 days.

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