◎原 著

Frizzled Activation by Wnt-1 Is Required for β-Catenin-T Cell Factor Dependent Transcription in Esophageal Cancer

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Abstract : Although, accumulation of nuclear and cytoplasmic β-catenin has been observed in ESCCs, mutation of APC and β-catenin are not found in ESCCs. Therefore, another mechanism for cytoplasmic β-catenin accumulation might exist in ESCCs. Materials and Methods : Human ESCCs cell lines and the 293 stable transfectants expressing Wnt-1, Wnt-5A, and Wnt-7A were cultured under standard conditions. The TOPFlash or FOPFlash reporter plasmids were transfected. Results : Transfected mutant β-catenin as well as an axin fragment harbing the GSK3β interaction domain, the latter a potent GSK3β inhibitor, both robustly activated pTOPFlash in ESCCs cells. When pTOPFlash/pFOPFlash reporters were transfected in ESCCs cell lines followed by co-cultivation with 293 cells that stably express Wnt-1, all cell lines except one demonstrated TCF mediated transcriptional. But, cells were co-cultured, Wnt-7A or Wnt-5A did not activate TCF mediated transcription in a cell number dependent fashion. Discussion; We report the activation of TCF promoter gene by external Wnt stimuli in ESCCs cells.

Key words : esophageal carcinoma, Wnt, TCF, β-catenin

Introduction

Human esophageal cancer is often an aggressive tumor with a poor prognosis (1). Recently, accumulation of nuclear and cytoplasmic β-catenin has been observed in esophageal squamous cell carcinoma (ESCC) (2, 3). It is involved in Wnt-Frizzled pathways that regulate cellular differentiation and proliferation (4, 5). In the absence of growth or differentiation signals, cytoplasmic β-catenin is low, since the protein is sequestered in complexes, which include the adenomatous polyposis coli (APC) protein, a

serine threonine glycogen kinase-3 (GSK-3) and conductin or axin, enabling degradation by proteasomes. Mutations in the APC or β-catenin genes result in constitutive activation of the β-catenin dependent transcription factor T-cell-factor (TCF) (6-10). The increased concentration of this protein in the cytoplasm favours its binding to the TCF/lymphoid-enhancer-binding-factor (TCF/LEF) family of DNA-binding proteins, and it subsequently translocates to the nucleus, where it induces transcription of specific genes stimulating tumor formation. Recently, cyclin D1 (11) and c-myc (12, 13) have been identified as target genes of β-catenin. This mechanism has been proposed as an important step in colorectal carcinogenesis.

Oncogene and tumor suppressor gene alterations (p53, cyclin D1, epidermal growth factor receptor) in ESCCs are observed frequently (14). Although, accumulation of nuclear and cytoplasmic β-catenin has been observed in ESCC, APC (2, 15) and β-catenin are not found in ESCC. Therefore, another mechanism for cytoplasmic β-catenin accumulation might exist in ESCC.

Wnt stabilizes cytoplasmic β-catenin and then β-catenin is translocated into the nucleus where it stimulates the expression of genes including c-myc, c-jun, fra-1, and cyclin D1 (16). In particular, a member of the human Frizzled gene family was found to be specifically expressed in ESCC (17). However, while Wnt is a candidate for the regulation of cytoplasmic β-catenin, little is known regarding the molecular relation of Frizzled, Wnt and β-catenin in ESCC.

To examine a potential role of Wnt in the development and progression in this disease, we show that Wnt-1 leads to activation of the β-catenin-TCF dependent gene transcription in ESCC cell lines.

Materials and Methods

Plasmids

pGL3-OT (pTOPFLASH) and pGL3-OF (pFOPFLASH) (gift of Dr. Bert Vogelstein) are luciferase reporter constructs containing wild type and mutated trimeric TCF binding sites, respectively (4). pCIneo-β-catenin XL and pCIneo-β-catenin △45 are expression constructs for wild-type and constitutively active mutant form of β-catenin, respectively (gift of Dr. Bert Vogelstein) (18). pCS2MT-axin GID1-2 and pCS2MT-axin GID L/P are constructs which express wild type and mutated GSK-3β interaction domains of *Xenopus laevis* axin, respectively (gift of Dr. Peter S. Klein) (19).

Cells

Human esophageal squamous cell carcinoma cell lines, TE1, TE3, TE5, TE8, TE10, TE11, TE12, and TE15, embryonic kidney 293 cells, and the 293 stable transfectants expressing Wnt-1, Wnt-5A, and Wnt-7A (gift of Dr. Elizabeth Wilder) were cultured under standard conditions, namely at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), and 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY).

Transfections and Luciferase Assay

Transfections were performed by using LipofectAMINE (Life Technologies, Inc., Grand Island, NY) according to the manufacturers in structions. At 24 h prior to transfection, 2.5 x 10^5 cells were seeded in 12-well plates. To assay the ability of Wnt to activate TCF/LEF transcription, TE series and EPC2 cells were transfected with 0.25 μg of the TOPFlash or FOPFlash reporter plasmid. At 36 hours after transfection, cells were harvested for luciferase

activity. All test variables were performed in triplicate. Luciferase assays were performed by using the Luciferase Assay System (Promega, Madison, WI) in Microtiter® Plate Luminometer (DYN-EX Technology, Chantilly, VA). β-galactosidase activities were determined by standard methods as a control for transfection efficiency.

Results

TCF mediated transcription is inactive in ESCC cell lines.

To determine TCF mediated transcriptional activity, we transfected pTOPFLASH and pFOPFLASH luciferase reporter constructs in several ESCC cell lines. None of the ESCC cell lines tested showed any appreciable TCF mediated transcriptional activity in the absence of external Wnt stimuli. The lack of constitutive activation of TCF mediated transcription in ESCC cell lines suggested that these cell lines are likely to carry intact genes for β-catenin, APC or axin whose mutations are frequently observed in certain subsets of cancers with activated β-catenin dependent TCF mediated transcription.

To exclude the possibility that ESCC cell lines lack the necessary transcriptional machinery including TCF transcription factors, we ectopically expressed several proteins known to potently activate β-catenin dependent TCF-mediated transcription. As shown in Fig. 1, transfected mutant β-catenin as well as an axin fragment harbing the GSK3β interaction domain, the latter a potent GSK3β inhibitor, both robustly activated pTOPFLASH in TE8 cells.

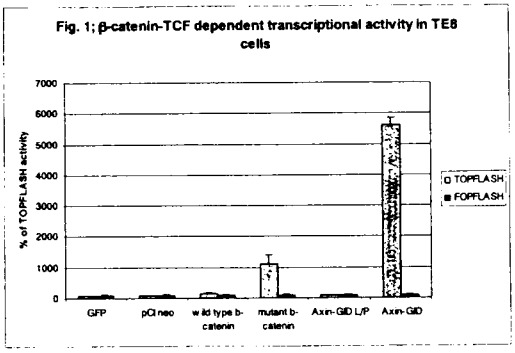

Figure 1. β-catenin-Tcf dependent transcriptional activity in TE 8 cells.

We investigated whether the machinery of Wnt-GSK-3β-Tcf/LEF pathway were present or not in ESCC cells. We transfected green fluorescent protein (GFP), empty vector (pCI neo), wild type β-catenin, mutant β-catenin, and wild type axin (Axin GID L/P) or mutant axin (Axin-GID) to TE8 cells. GFP and pCI neo served as transfection controls. Transfection of mutant β-catenin or mutant axin activated TCF/LEF reporter gene compared to wild type. These results indicated that ESCC cell had the complete machinery of the pathway.

Regulation of TCF mediated transcriptional activity by Wnt-1 in ESCC cell lines.

To investigate the possible activation of Wnt signaling pathway in ESCC cell lines, we tested the effect of Wnt-1, Wnt-5A, and Wnt-7A on TCF mediated transcriptional activity. When pTOPFLASH/pFOPFLASH reporters were transfected in ESCC cell lines followed by co-cultivation with 293 cells that stably express Wnt-1, all cell lines demonstrated TCF mediated transcriptional except TE12 (Fig. 2). Transcriptional activation was not observed when the transfected cells were co-cultured with parental 293 cells. Therefore, the activated TCF mediated transcription in ESCC cell lines is attributable to the expressed Wnt-1 in the medium. Consistent with this idea, TCF mediated transcription in TE8 cells was activated depending on the cell number of the co-cultured stable transfectants (Fig. 3). When TE8 cells were co-cultured, Wnt-7A but not Wnt-5A weakly activated TCF mediated transcription in a cell number dependent fashion (Fig. 3).

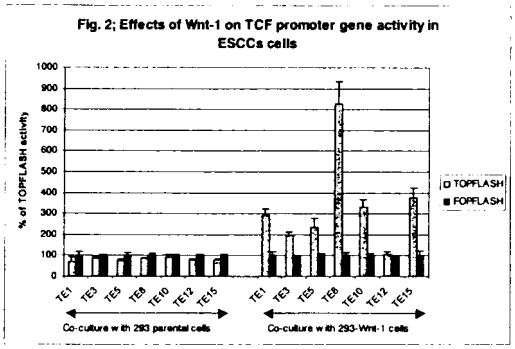

Figure 2. Effects of co-cultures with 293-Wnt-1 or 293 parental cells on β-catenin-TCF dependent transcriptional activity in ESCCs cells.

ESCC cells were co-cultured with Wnt stable transfected 293 cells or parental 293 cells. Two forms of TCF/LEF promoter were used, one consisting of TOPFLASH (a reporter plasmid containing multiple copies of wild type TCF binding sites), the other of FOPFLASH a reporter plasmid containing mutant TCF-binding site). β-galactosidase activities were determined by standard methods as a control for transfection efficiency. The ratio of reporter luciferase activity to control β-galactosidase activities is indicated. All experiments are expressed as mean±S. D. of triplicate cultures.

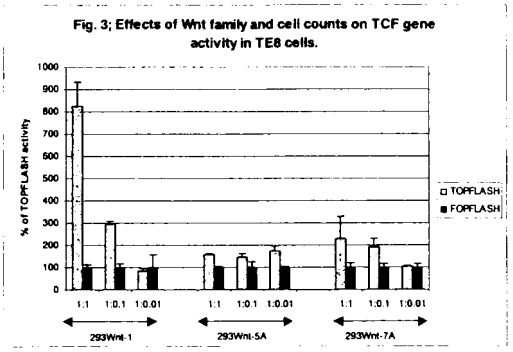

Figure 3. Effects of Wnt on TE8 cell

TE8 cells were co-cultured with 293Wnt 1, 293Wnt5A or 293Wnt7A and transfected with 0.5 μg of wild-type (TOPFLASH) or mutant-type (FOPFLASH) TCF promoter expression plasmid. Cell number ratio between TE8 and 293Wnt-1, 5A or 7A were 1 : 1, 1 : 0.1 or 1 : 0.01, respectively. The ratio of reporter luciferase activity to control β-galactosidase activities is indicated. All experiments are expressed as mean±S. D. of triplicate cultures.

Discussion

We report the activation of TCF promoter gene by external Wnt stimuli in ESCCs cells. The Wnt/Frizzled signaling pathway stabilizes β-catenin/APC complexes and increases free intracellular pools of β-catenin by inactivation of GSK-3 (7). We showed that Wnt-1 activated the TCF promoter gene. To our knowledge, this is the first report of functional TCF/LEF activation by Wnt-1. Our results indicate the possibility that Wnt has potential role in development and progression in ESCC. Inactivation of APC function induces stabilization of the β-catenin protein. More recently, the stabilized free β-catenin has been found to bind to high mobility group box-containing transcription factors including LEF (6) and TCF (4) and the complexes translocate to the nucleus. The nuclear β-catenin is thought to act in conjunction with the LEF/TCF transcription factors to activate gene expression associated with cell proliferation or inhibition of apoptosis (20).

Activated Frizzled receptors induce the stabilization of the cytoplasmic component β-catenin by blocking the function of a multiprotein β-catenin destruction complex (21). This complex consists of the scaffolding component axin, or the related conduction. These bind to the tumor suppressor protein APC, GSK3β and β-catenin through separate domains. In the absence of a Wnt signal, GSK3 mediates the phosphorylation of β-catenin and the ubiquitination machinery, and finally degraded in proteasomes . In the presence of Wnt, the cytoplasmic phosphoprotein dishevelled is activated and interferes with the β-catenin destruction complex. GSK3 activity is inhibited, and the phosphorylation of β-catenin is blocked. Hypophosphorylated β-catenin is no longer degraded and accumulates

in the cytoplasm and the nucleus.

Wnt-1 was identified as a preferred integration site for mouse mammary tumor virus in breast adenocarcinoma (22). In addition, Wnt-1 is able to transform cultured mammary epithelial cell lines (22) and transgenic mice expressing Wnt-1 in the mammary gland develop mammary epithelia hyperplasias and adenocarcinomas (23). However, in these transgenic studies, it is clear that Wnt-1 expression alone is insufficient for malignant (24).

A long-standing difficulty in studying the action of mammalian Wnt genes has been the inability to isolate their products in the soluble form that demonstrates functional activity (25). Our results indicate that Wnt-1 protein can act on target cells externally as a soluble secreted factor.

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食道癌細胞におけるWnt のベータカテニンTCF経路の活性化の検討

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背景：食道癌細胞にベータカテニンの蓄積があることは報告されているが，大腸癌とことなり，ベータカテニン，Axin，APC変異の報告がなく，そのメカニズムは不明である。
方法：８種類の食道癌細胞株において，３種類のWntの刺激によるベータカテニンTCF系の活性化への影響をTCFプロモーターを持つルシュフフェラーゼプラスミドを癌細胞にトランスフェクションして検討した。
結果：代表的な食道癌細胞は変異ベータカテニン，変異APC，変異Axinのいずれも，TCFの活性化をおこした。８種類中７種類の癌細胞はWnt1により量依存性のTCFの活性化が認められたが，Wnt５A，Wnt７Aでは活性化が認められなかった。
結語：食道癌の進展にWnt1の関与が示唆された。