Involvement of Deregulated Epiregulin Expression in Tumorigenesis in Vivo through Activated Ki-Ras Signaling Pathway in Human Colon Cancer Cells

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Abstract

To identify the genes located downstream of the activated Ki-Ras signaling pathways in human colon cancer cells, a PCR-based cDNA subtraction library was constructed between HCT116 cells and HCT116-derived activated Ki-ras-disrupted cells (HKe3). One of the genes in HCT116 that was evidently up-regulated was epiregulin, a member of the epidermal growth factor family that is expressed in many kinds of human cancer cells. HKe3-stable transfectants expressing activated Ki-Ras regained over-expression of epiregulin. To further elucidate the biochemical structure and significance of epiregulin expression in tumorigenesis, HKe3-stable transfectants expressing epiregulin (e3-pSE cells) were established. Epiregulin existed as highly glycosylated membrane-bound forms, and TPA rapidly induced ectodomain shedding of epiregulin. Furthermore, the conditioned medium of e3-pSE cells showed more DNA synthesis for 32D cells expressing epidermal growth factor receptor (DER) cells than that of HKe3. Although anchorage-independent growth in soft agar was not observed for e3-pSE cells, tumorigenicity in nude mice was observed evidently, and their growth rate was correlated with each amount of exogenous epiregulin expression. These results suggested that activated Ki-Ras will be one of the factors contributing to the over-expression of epiregulin in human colon cancer cells, and that epiregulin will play a critical role in human tumorigenesis in vivo.

Introduction

Ras has been implicated in controlling cell proliferation, differentiation, and apoptosis. Activated Ki-ras oncogenes were found in the majority of exocrine pancreas carcinomas and in a high frequency of colorectal tumors (1–5); however, the activated Ki-Ras-mediated signaling pathway still remains obscure. Earlier, we established HCT116-derived cells in which activated Ki-Ras signaling pathways still remains obscure. Here, we obtained evidence that activated Ki-Ras-mediated signaling induces epiregulin expression in human colon cancer cells, and that epiregulin is involved in tumorigenesis in vivo.

Materials and Methods

cDNA Subtraction Library and Isolation of Differentially Expressed Genes. Differentially expressed genes between HCT116 cells and HKe3 cells were identified by a PCR-based cDNA subtraction library (PCR-Select cDNA Subtraction Kit; Clontech, Palo Alto, CA) using poly(A) RNA from exponential-growth condition of HCT116 and HKe3. We screened 288 clones and finally got several genes strongly expressed in HCT116. Full-length epiregulin cDNA was isolated by conventional plaque hybridization from the HCT116 cDNA library constructed with a ZAP-cDNA Synthesis and Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA) using an epiregulin cDNA fragment as a probe. DNA sequences were determined by ABI Prism Big Dye Terminator Cycle Sequencing (Applied Biosystems) with an ABI 377 Sequencer.

Northern Blot. Cells were cultured in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS or serum-starved for 24 h in 10% CO2 at 37°C. Cells were collected with phosphate-buffered saline, and then total RNA was isolated by ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer’s protocol. Total cellular RNA (20 μg) was separated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to nylon membrane (Pall, East Hills, NY), and the filters were hybridized with probes for epiregulin, EGF, transforming growth factor-α, HB-EGF, and β-actin cDNAs, as described (10).

HKe3-derived Stable Transfectants. Activated Ki-ras exon 4B (Gly13Asp) cDNA with the HA-epitope tag at the N-terminal region of Ki-ras was made by reverse transcription-PCR using total RNA from the HCT116-derived normal Ki-ras-disrupted cells (6) and then subcloned into pBluescript SK plasmid (Stratagene, La Jolla, CA). Nucleotide sequences of HA-MKRas were confirmed by sequencing, and then the HM-MKRas fragment was subcloned into the pSI expression vector (Promega, Madison, WI). Linearized pSI-HA-MKRas (20 μg of DNA) and pcDNA6 (2 μg of DNA; Invitrogen, Groningen, the Netherlands) were cotransfected into HKe3 cells by the calcium phosphate method. Cells maintained in DMEM with 10% FCS were split after 2 days, and cells were selected with 10 μg/ml blasticidin (Invitrogen). Colonies surviving in selection media for 14 days were picked up under the microscope and expanded and characterized for the expression of HA-MKRas by Western blot using anti-HA antibody (Roche). Finally, HKe3 cells expressing HA-MKRas (e3-MKRas#9 and e3-MKRas#14) were established. Epiregulin cDNA with HA at the COOH-terminal region of epiregulin was made from isolated human epiregulin cDNA using PCR. The epiregulin-HA fragment was subcloned into the pSI vector, and then pSI-epiregulin-HA and pcDNA6 were cotransfected as described above, and HKe3-stable transfectants expressing epiregulin-HA (e3-pSE#26, e3-pSE#36, and e3-pSE#65 cells) were established. Western blot was done using the enhanced chemiluminescence system (Amersham-Pharmacia) as described previously (10).

Surface Biotinylation and Immunoprecipitation. Biotinylation of cell surface proteins was done using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) following the manufacturer’s protocol. Cells of HKe3 and e3-pSE#26 in human adult tissues (12–14). Although epiregulin can be a potent pan-ErbB ligand (15), its biological significance in tumorigenesis still remains unknown. Here, we obtained evidence that activated Ki-Ras-mediated signaling induces epiregulin expression in human colon cancer cells, and that epiregulin is involved in tumorigenesis in vivo.
(4 × 10^3) were collected and incubated with 1 ml of reaction buffer containing 0.5 mg of biotin for 1 h at room temperature. Then, cells were lysed with 1 ml of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 10 mM orthovanadate, and complete protease inhibitor cocktail (Roche)], and the supernatants were collected into 1.5 ml tubes. The supernatants were precleared with protein G-Sepharose (Amersham-PharMingen), and then anti-HA antibody was added at the final concentration of 2 μg/ml and rotated overnight at 4°C, then protein G-Sepharose was added for 2 h. Protein G-Sepharose complexes were boiled for 10 min, and the supernatants were run in 14% SDS-PAGE. Western blots were done using SA-HRP (Amersham-PharMingen).

TPA Stimulation. Cells were serum-starved for 24 h and then stimulated with 50 ng/ml TPA. Cells were collected at the indicated times, lysed with RIPA buffer, and then immunoprecipitated as described above. Western blots were done with anti-HA antibody and anti-Rat-immunoglobulin-HRP (PharMingen).

**Conditioned Media and Measurement of DNA Synthesis.** Murine 32D cells and DER cells, 32D cells expressing human EGFR (16), were maintained in RPMI 1640 medium supplemented with 10% FCS and 5% WEHI-3 cell-conditioned medium as a source of IL-3. HCT116, HKe3, e3-pSE#6, e3-pSE#65, and e3-pSE#65 cells were plated in 24-well plates at a density of 1 × 10^5 cells/well and cultured in 50 μl/well RPMI with 10% FCS for 48 h. The conditioned media were collected and preclarified by centrifugation at 1,200 × g for 10 min. DER cells or 32D cells at a density of 2 × 10^6 in 50 μl of RPMI 1640 containing 10% FCS were mixed with 450 μl of the conditioned media samples in 24-well plates. After culturing for 30 h, the degree of DNA synthesis was determined by measuring the incorporation of [3H]-thymidine for 4 h.

**Assay for Tumorigenesis.** Soft agar colony formation assays were done as described (6), and photographs were taken at 3 weeks after seeding. Cells (5 × 10^5) were s.c. injected into the flanks of athymic nude mice, and tumor volume (mm^3) was determined by using the standard formula a^2 × b/2, where a is the width and b is the length of the horizontal tumor diameter every week. Some athymic nude mice were used for each cell, and the data were represented by the mean value.

**Results and Discussion**

To identify the genes located downstream of the activated Ki-Ras-mediating signals, a PCR-based cDNA subtraction library was constructed between HCT116 cells and HCT116-derived activated Ki-ras-disrupted clone cells (HKe3) through homologous recombination (6). The epiregulin cDNA fragment was detected in 288 clones examined from this subtracted cDNA library. Epiregulin cDNA was isolated from the HCT116 cDNA library, and its DNA sequencing showed no mutation of the epiregulin-coding region (data not shown). To confirm the differential expression of epiregulin between HCT116 cells and HKe3 cells, Northern blot was done under exponential, serum-starved, and TPA-stimulated conditions. Epiregulin was strongly expressed in HCT116, whereas it was rarely observed in HKe3 under exponential and serum-starved conditions (Fig. 1). However, TPA induced epiregulin expression in HKe3 (data not shown), suggesting that HKe3 cells contains the epiregulin genome. To elucidate further the relationship between activated Ki-Ras and epiregulin expression in HCT116, HKe3-stable transfectants expressing the activated Ki-Ras (Gly13Asp) mutation, which is the same as the Ki-ras mutation of HCT116, were established; and these cells showed the same morphology as HCT116 (data not shown). HKe3 transfectants expressing mutant Ki-Ras (e3-MKRas#9 and e3-MKRas#14) evidently expressed epiregulin (Fig. 1). Taken together, these results suggested that epiregulin was overexpressed through the activated Ki-Ras signaling pathway in HCT116.

To determine the biochemical character and the biological significance in tumorigenesis of epiregulin, we established the HKe3-stable transfectants expressing HA-epiregulin. Western blot showed that epiregulin existed as several modified forms, and e3-pSE#26, e3-pSE#65, and e3-pSE#36 were low-, middle-, and high-epiregulin expressing clones, respectively (Fig. 2A). To elucidate the modification of epiregulin, surface biotinylation of epiregulin was done on HKe3 and e3-pSE#26, and then by immunoprecipitation with anti-HA antibody, the tag of which was inserted at the COOH-terminal region of epiregulin. Only the molecular size of about M, 34,000 was detected with SA-HRP, indicating that this product was the membrane-bound form and the lower migrating bands were not present at the cell-surface (Fig. 2B). The membrane-bound forms were modified with N-linked glycosylation (data not shown). Furthermore, TPA rapidly induced ectodomain shedding of epiregulin, resulting in the disappearance of the membrane-bound form of epiregulin and the accumulation of the cytoplasmic tail (Fig. 2C). These results, together, suggested that epiregulin exists as membrane-bound forms and as mature secreted forms.

Next, we investigated whether secreted epiregulin from HKe3 transfectants expressing epiregulin can stimulate the growth of DER cells (16). The growth of 32D cells is dependent on IL-3. Dependent on the expression level of epiregulin, the conditioned medium from HKe3 transfectants expressing epiregulin stimulated EGFR in DER cells, resulting in the DNA synthesis without IL-3 (Fig. 3), whereas 32D cells demonstrated little DNA synthesis when the conditioned medium was used. Furthermore, transforming growth factor-α, EGF, or heparin-binding EGF-like growth factor were rarely expressed in HCT116, HKe3, or e3-pSE cells (data not shown). These results, together, suggested that secreted epiregulin will be one of the target molecules involved in tumorigenesis through the activated Ki-Ras-mediated pathway in HCT116 cells.

Tumorigenicity in vitro and in vivo, assayed by soft agar colony formation and tumor growth in nude mice, respectively, were dramatically reduced in HKe3 cells when compared with those of HCT116 cells (6). To assess the functional role of epiregulin in vitro, anchorage-independent growth was assayed on HKe3 cells expressing epiregulin. HCT116 cells showed obvious colonies in soft agar, whereas none of the clones made evident colonies (Fig. 4A; data not shown). However, HKe3 cells expressing epiregulin showed a higher growth rate of tumors in nude mice compared with that of HKe3 cells, and the growth rates were correlated with the amount of epiregulin expression in each (Fig. 4B, Fig. 2B). Although the growth rate of HKe3 cells expressing exogenous epiregulin was lower than that of HCT116 cells (Fig. 4B), these results suggested that epiregulin will be critically involved in tumorigenesis in vivo.

The function of activated Ki-Ras seems to be different from that of other members of the Ras family (17–19), and how activated...
Ki-Ras is involved in tumorigenesis in human cancer still remains unclear. We have demonstrated previously that activated Ki-Ras is involved in the up-regulation of VEGF and the urokinase-type plasminogen activator receptor, leading to angiogenesis and lami-

Fig. 2. HKe3-derived stable transfectants expressing epiregulin-HA. A, Western blot with anti-HA antibody. Epiregulin-HA was detected in several modified forms (arrow, arrowheads). B, surface biotinylation. HKe3 cells and e3-pSE#26 cells were surface-biotinylated and then immunoprecipitated with anti-HA antibody. The immunoprecipitated products were detected by SA-HRP (left) and anti-HA antibody (right). M, 34,000 bands (arrow) were only cell-surface proteins, and the smaller bands (arrowheads) did not exist at the cell surface. C, TPA-induced ectodomain shedding of epiregulin. HKe3 cells were serum-starved for 24 h and then stimulated with TPA (50 ng/ml). Proteins were extracted at the indicated time points and lysed with RIPA buffer. Immunoprecipitation was done with anti-HA antibody and blotted with anti-HA antibody. IP, immunoprecipitation; WB, Western blot; SA, streptavidin; IgL, immunoglobulin light chain.

Fig. 3. Epiregulin activities in the conditioned media of the HKe3 cells expressing exogenous epiregulin. The growth factor activities of the conditioned media were determined by measuring the amount of DNA synthesis. DER cells (2 × 10^5) were incubated with [3H]-thymidine for 4 h. The amounts of [3H]-thymidine incorporated into the DNA were measured by a scintillation counter. Data show the mean ± SD in duplicate results, and similar results were obtained in two independent experiments. In the case of 32D cells, the degrees of DNA synthesis were <1,000 cpm in all cases of the conditioned media samples.

Fig. 4. Assays of tumorigenicity for HKe3-stable transfectants expressing epiregulin-HA. A, anchorage-independent growth determined by soft agar colony formation. Cells (5 × 10^3) were seeded in 0.3% top agarose on 0.6% base agar as described (6), and the photographs were taken at 3 weeks after seeding. B, tumorigenicity in athymic nude mice. Cells (5 × 10^5) were injected subcutaneously into the flanks of nude mice. Tumor volume (mm^3) was estimated by the formula described in "Materials and Methods."

References


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