学 位 論 文 の 要 旨

氏名　FARZANA BINTE RAHMAN

学　位　論　文　名　Fibroblast Derived HB-EGF Promotes Cdx2 Expression in Esophageal Squamous Cells

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著　者　名　Farzana Binte Rahman, Yasunori Kadowaki, Shunji Ishihara, Hiroshi Tobita, Hiroshi Imaoka, Hiroyuki Fukuhara, Md. Monowar Aziz, Kenji Furuta, Yuji Amano, Yoshikazu Kinoshita

論 文 内 容 の 要 旨

INTRODUCTION

The molecular basis of attaining columnar phenotype in Barrett's esophagus is poorly understood. One hypothesis states that factors locally produced by cells of mesenchymal origin in chronic reflux esophagitis induce metaplastic transformation. Several lines of evidence support the influences of stromal fibroblasts on degenerative changes of the epithelial environment, notably in salivary glands, mammary glands, uninary bladder, and skin. Less is known about the pattern of fibroblast secretory proteins or factors that modulate the growth and differentiation of adjacent progenitor or matured epithelial cells in the esophagus. The present study was performed to elucidate the factors secreted from fibroblasts in response to acid that cause columnar phenotype in adjacent squamous epithelium.

MATERIALS AND METHODS

In the present study, we employed an *in vitro* system by growing human embryonic fibroblast cells OUMS-36T-6 in acidified media (pH range of 3.5, 4, 5, 6, or 7) for 20 minutes, followed by replacing the acidified media to normal one and then allowed to culture for up to 72 hours. The cell viability was assessed by MTT assay while the microscopic examination was

done to determine whether their morphology was affected. To determine the status of gene expression after a single pulse of acid stimulation to fibroblast cells, microarray analysis (Sentrix Human-6 Expression BeadChips were used) was done using mRNA from samples of 2 hours after 20 minutes of acid treatment. The microarray results were further confirmed by quantitative real time RT-PCR and ELISA techniques. In order to support our *in vitro* findings, whether acid induces HB-EGF expression in fibroblast cells, we also performed immunohistochemistry for HB-EGF expression in biopsy specimens from 12 patients with reflux esophagitis as an additive approach to show as an *in vivo* evidence. The protocol was approved by the ethical review committee of Shimane University School of Medicine and all the subjects had given their informed consents before studies were performed. In the next part of our study, we evaluated the role of recombinant HB-EGF to induce columnar marker gene expression in human esophageal squamous epithelial cell line, HET1A and human esophageal adenocarcinoma cell line OE-33. Cdx2, cytokeratin7 (CK7), CK8, CK18 and villin were chosen as columnar marker gene and their expression at mRNA and protein level was checked by quantitative PCR, immunofluorescence and western blot in HB-EGF treated HET1A cells. Human Cdx2 promoter of 960bp (-925 to + 34) size was cloned into the PGL3 reporter vector. From this parent promoter construct, deleted length truncated promoters were constructed by PCR and transfected into HET1A cells. Their activity was measured by Dual-Luciferase reporter assay. Two potent transcription factor, AP-1 (-162 to -173) and NF-κB (-79 to -88) binding sites were revealed at the proximal end of Cdx2 promoter and Site-directed mutagenesis was performed for incorporation of mutations at those sites. Finally, an EGFR specific siRNA and pharmacologic inhibitor, AG1478 was used to confirm that the HB-EGF effects were mediated by EGFR pathway in HET1A cells.

RESULTS AND DISCUSSION

The results showed that the growth, morphology and viability of the fibroblast cells were maintained as normal at the low pH media, while these were significantly affected at pH below 5. Based on the growth curve and morphology under light microscope, we selected pH 5 as the optimal acidic pH condition for survival of the fibroblast cells and performed microarray

analysis subsequently. From a large number of up regulated genes in microarray analysis, we selected some potent growth factors e.g. FGF6, HB-EGF, PDGFA, PDGFB, NELL1, TGFB3 and cytokines e.g. IL8, CSF2, IL1β, CXCL5, stromal cell derived factor 1α, TNFSF12 that are usually involved in oncogenicity, inflammation, differentiation, and transformation. We confirmed their upregulation at the mRNA and protein level by quantitative real time RT-PCR and ELISA respectively. Studies with human subjects showed that two of the reflux esophagitis biopsy specimens with mucosal breaks were found to be positive for HB-EGF staining in the loosely adherent fibroblast cells, as compared to that of healthy volunteers. Cdx2, an intestine specific transcription factor and member of the caudal related homeobox gene family is important in the differentiation of esophageal and gastric epithelium into intestinal type epithelium, the effect of HB-EGF on Cdx2 expression has been investigated elaborately. We noted that, recombinant HB-EGF promoted Cdx2 expression in HET1A and OE-33 cells both at mRNA and protein level in a dose dependent manner, with optimum induction at 1ng/mL of HB-EGF stimulation. Since gene expression is controlled by regulatory units, we investigated the effects of HB-EGF on Cdx2 promoter function. HB-EGF caused considerable upregulation (~3.5 fold) of the Cdx2 promoter in HET1A transfected cells, while its effects were gradually declined with decreases in size of the parent promoter. Introduction of mutation on AP-1 and NF-κB consensus sites significantly diminished HB-EGF activity on Cdx2 promoter. Other columnar epithelium marker CK7, CK8, CK18, villin expression was also induced by HB-EGF stimulation. Cells treated with EGFR specific pharmacologic inhibitor AG1478 or siRNAs dramatically reduced the effects of recombinant HB-EGF on Cdx2, CK7 and villin gene expression, as well as the activation of AP-1, NF-κB, while those were found to be unaffected in non-target specific control siRNA-treated conditions.

CONCLUSION

Acid induced HB-EGF in fibroblast cells may induce several columnar marker gene expressions in esophageal squamous epithelial cells.