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（8）創傷治癒過程に於ける制御機構の研究

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理事長 中島 章 殿

研究発表中または研究中の本人のスナップ写真、及び発表論文のコピーを添付

1．研究者氏名 呂月予

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研究テーマ 創傷治癒過程における制御機構の研究

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有) ・ 無 (学会名・内容)

1．Combined Meeting of American Pancreatic Association and International Association of Pancreatology ２０００年１１月 Chicago USA
EXPRESSION AND LOCALIZATION OF LUMICAN IN HUMAN PANCREATIC CANCER

2．第５６回日本癌学会総会 ２０００年１０月 日本 横浜
大腸癌における Fibroblast growth factor-10(FGF-10)の発現の検討

3．第３０回創傷治癒学会 ２０００年８月 日本 東京
心虚血再灌流モデルにおける lumican の発現とその局在の検討

4．第１１回日本医科大学 外国人留学生研究会 ２０００年 １１月
膵臓癌における lumican の発現とその役割の検討

(2) 学会誌等に発表した論文 有 ・ (無) (雑誌名・論文名)

3．今後の研究計画

今後は研究対象を広げ、創傷治癒と癌組織増殖過程における細胞外基質とプロテオグリカンの局在と発現につき比較研究を行予定である。
正常の大腸組織においてプロテオグリカンの一種の lumican messenger RNA の発現と局在を検討し、大腸癌組織、正常大腸組織と人工肛門組織での lumican タンパクの発現を検討する. 大腸癌組織と人工肛門組織における lumican mRNA 発現細胞の比較検討を行う。大腸癌組織、正常大腸組織、人工肛門創と比較し、それぞれの病態における Lumican の役割を明らかにする.

4．研究指導者の意見

吉君は日本医科大学病理学第二教室の研究生として一般病理学研修とともに免疫組織化学的手法と分子病理学的手法を用いた研究に、精力的に取り組んでおります。人工肛門創の治癒過程における促進制御機構についての研究では、Transforming growth factor-β (TGF-β)と Hepatocyte growth factor (HGF)の役割につき検討し、第２８回、２９回の創傷治癒学会と第８８回病理学会総会、第７回アジア大腸肛門学会（北京）にて報告しております。これらの成果の一部は Wound Repair and Regeneration 誌にまとめております (WOUND REP REG 2000,8; 59-67)。

１９９９年より同君は膵臓癌細胞の増殖と浸潤における細胞外マトリックスの役割と制御機構の研究を行っております。その成果を２０００年１１月に米国シカゴで開かれた、Combined Meeting of American Pancreatic Association and International Association of Pancreatology で報告し、その内容は評価を受け Research Travel Award を受賞しております。今後はさらに研究の進歩が期待できるものと思われます。

研究指導者氏名 石渡俊行 ㊞

5．研究報告

別紙形式により、報告本文4000字以上(英文は2600語以上)で報告して下さい（枚数自由・ワープロ使用）
タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入、使用文字はタイトル１３ポイント、その他は１０ポイント、日本語は明朝体を使用して下さい。
研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。
論文発表に当っては、*日中医学協会助成事業－日本財団助成金による*旨を明記して下さい。

Differential expression of hepatocyte growth factor and its receptor (c-Met) in a rat artificial anus model

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要旨

人工肛門は皮膚の重層扁平上皮と大腸腺上皮という異なった種類の上皮細胞が吻合し、特異的な創傷治癒過程を示すことが知られている。一方 Hepatocyte Growth Factor (HGF) は腫瘍の増殖、進展や創傷治癒過程において、細胞増殖、組織の再生、修復に重要な役割を果たしていることが報告されている。消化管においては、胃潰瘍の治癒過程で胃の腺上皮の再生増殖と腺管の管腔形成に関与することが示されている。HGF は主に間葉系細胞で産生され、HGF activator により活性化された後、細胞膜に局在する HGF receptor (c-Met) を介し上皮系細胞に作用する増殖因子である。今回ラットの人工肛門創モデルを作成し、術後１、３、７、１４日目に人工肛門組織を摘出した。HGF と c-Met の局在とそれぞれの messenger RNA の発現動態および HGF と c-Met の役割につき免疫組織化学法、Competitive RT-PCR 法および In situ hybridization 法で検討を行った。H.E 染色では術後１日目に重層扁平上皮と腺上皮細胞の増殖がみられ、吻合部の間質にフィブリンと軽度の炎症細胞浸潤が認められた。３日目では吻合部にフィブリンと炎症細胞浸潤が著明で、間質には fibroblast と血管の増生が認められた。７日目には吻合部周囲の腺管構造が正常の腺上皮と同様の形態を示し、肉芽組織内には collagen fiber の出現もみられた。１４日目には間質内に広範囲に線維化がみられ、炎症細胞浸潤も吻合部周囲に限局していた。Competitive RT-PCR 法では術後に HGF mRNA の発現量が術後１日から 14 日まで徐徐と増加するのに対し C-Met mRNA の発現量は術後１日と７日目で増加が認められた。In situ hybridization 法では HGFmRNA は主に間質の fibroblast に、c-Met mRNA は吻合部の重層扁平上皮細胞と腺上皮細胞の胞体内に発現が確認された。Western blot 法では人工肛門組織で６２kd の成熟型の HGF のバントが認められるのに対し、無処置対照の皮膚と大腸組織では成熟型 HGF のバントは認められなかった。HGF 蛋白は大腸粘膜上皮と皮膚の扁平上皮、肉芽組織の中の fibroblast, 血管内皮細胞と炎症細胞に局在がみられた。術後３日目で再生した吻合部の大腸腺管に強い c-met 蛋白の陽性像がみられ。腺管再生上皮と吻合部の肥厚した表皮細胞には豊富な c-met の局在が見られた。

以上の結果よりラットの人工肛門創傷治癒過程では、重層扁平上皮と腺上皮の増殖が術後早期より起こり、１週後には再生腺管が形成されることが確認された。HGF は治癒過程早期では人工肛門組織に産生が少なく増殖期以降は増加しており、肉芽組織の形成に伴う線維芽細胞や血管内皮細胞が重要な役割を果たしていることが示唆された。 創傷治癒過程早期と上皮の形態形成期では HGF receptor が皮膚の重層扁平上皮と大腸の腺上皮細胞に豊富に局在することが確認され、HGF/c-Met 系の作用が人工肛門の治癒過程において皮膚と大腸の両上皮の再生修復に重要であることが示された。

Key words: HGF, c-Met, artificial anus, in situ hybridization, competitive RT-PCR

Aim

Hepatocyte growth factor (HGF) is a hepatotrophic factor, initially purified from rat platelets[1] that promotes liver regeneration. HGF has been shown to stimulate the growth of various epithelial cells, including epithelial cells of the gastrointestinal tract[2], renal tubular cells[3], epidermal melanocytes[4], and keratinocytes[5], and furthermore the proliferation of endothelial cells[6]. On the other hand, HGF also promotes the organization and reconstruction of tissues, suggesting that it might play an important role in the process of tissue repair[7]. It is known to be predominantly produced in mesenchymal cells, including fibroblasts[8], Ito cells[9], Kupffer cells and endothelial cells[10]. The HGF receptor has been identified as the product of the proto-oncogene, c-Met, which has been reported to be a specific transmembrane receptor[11,12]. C-Met is mainly localized in epithelial cells and endothelial cells, suggesting that HGF/c-Met signals play a role in mesenchymal-epithelial interactions[13]. Clinically, an artificial anus is constructed as a curative and preservative operation for colorectal cancer. Construction of an artificial anus is surgically established operation, however it shows a unique regenerative process. In the artificial anus, two different histological types of epithelial cells (the squamous cells of the skin and colonic ductal cells) intermingle with each other. Some of the squamous-ductal junctions known in the normal human body are the esophago-gastric junction (EC-junction), junction between the cervix and body of uterus, and the anorectal junction. The repair process at artificial squamous-ductal epithelial junctions and the role of growth factors have not been clarified. Therefore, in the present study, we examined the localization and mRNA expression of HGF and c-Met in the colonic and skin epithelial cells in an artificial anus. Furthermore, we also attempted to clarify the main epithelial target of HGF in this regenerative condition. We now report that HGF is overexpressed predominantly in the stromal component (macrophages, fibroblasts and endothelial cells) and c-Met and its mRNA are overexpressed predominantly in the regenerating squamous cells of the skin in the rat artificial anus.

Materials and Methods

Animals : Wistar male rats weighing 190-210 g were used for all the experiments (Sankyo Co., Tokyo, Japan). An artificial anus was surgically constructed with its opening at the lower left quadrant of the rat abdomen. Animals were sacrificed 1, 3, 7 or 14 days after the operation and the artificial anus was excised along with the abdominal skin The study was approved by the Animal Ethics Committees of the Nippon Medical School, Japan.

Western blot analysis : The artificial anal tissue specimens from the rats were solubilized in lysis buffer. The proteins extracted were subjected to SDS-PAGE and transferred to IPVH membranes, which were then incubated for 60 min with anti-HGF α antibody. The membranes were then washed and incubated with secondary biotinylated anti- goat IgG antibody for 60 min, and thereafter with the peroxidase-avidin complex. After washing, antibodies were visualized by enhanced chemiluminescence.

Immunohistochemistry : The highly specific goat anti-human HGF α antibody used for Western blotting, and goat anti-mouse c-Met polyclonal antibodies were utilized for immunohistochemistry. Paraffin-embedded sections (3 m) were subjected to immunostaining using the streptavidin-peroxidase technique.

Competitive reverse transcriptase polymerase chain reaction (Competitive RT-PCR) :
Total RNA extraction and cDNA synthesis were performed. Competitive RT-PCR was performed according to the protocol of the PCR MIMIC Construction Kit (Clontech). Before cDNA synthesis, total RNA was run on denatured gel to eliminate the possibility of RNA degradation, then used for reverse transcription. Competitive RT-PCR was repeated at least two times from initial transcription step. Total RNA not subjected to reverse-transcription was employed as the negative control.

In situ hybridization : To make a riboprobe for in situ hybridization, a 512-bp cDNA fragment of HGF and a 320-bp cDNA fragment of c-Met generated by RT-PCR amplification from rat liver RNA or rat heart were subcloned into the pGEM-T vector. The probes were labeled with digoxigenin-UTP by SP6 or T7 RNA polymerase using the DIG RNA Labeling Kit. In situ hybridization was performed.

Results

Histological findings : On the day 1 after the operation, fibrin deposition, neutrophil and lymphocytic infiltration were noted at the site of anastomosis (Fig. 1A, arrow). Colonic ductal cells showed regeneration and a simple layer of colonic epithelium proliferated at that site (Fig. 1A arrowheads). Fresh granulation tissue with fibroblasts, macrophages, capillaries and lymphocytes was formed at the site of anastomosis three days after the operation (Fig. 1B). The skin and colonic epithelium came into contact on day 7 after the surgery (Fig. 1C, arrow). The regenerated ductal cells showed common duct-like structures (Fig. 1C, arrowheads) and the squamous epithelium of the skin exhibited proliferation and acanthosis. Fibrotic changes with macrophages and lymphocytic infiltration were recognized in the granulation tissue (Fig. 1C). At fourteen days after the operation, regenerated ductal cells of the colon and squamous cells of the skin intermingled closely with marked fibrosis (Fig. 1D). Focal inflammatory cell infltration was noted under the ductal epithelium and the ductal cells showed inflammatory change (Fig. 1D, arrowheads).

Western blot analysis : HGF is synthesized as pro-HGF, and then digested by a HGF activator to the mature form of HGF. Only the HGF has any biological effects on epithelial cells. To confirm whether mature HGF is localized in the rat artificial anus, Western blot analysis was performed. The normal skin and colonic tissue did not show any bands, corresponding to pro-HGF or mature HGF. In contrast, all the tissue specimens obtained from the artificial anus exhibited a positive band at 62 Kd, corresponding to the a chain of the mature HGF protein of the rat(20) (Fig. 2).

Immunohistochemistry : To confirm the localization of HGF and the HGF receptor (c-Met), immunohistochemistry was performed. In the normal skin and colonic tissues. In the artificial anus, the regenerated ductal epithelium of the colon and the squamous epithelium of the skin exhibited mild to moderate HGF immunoreactivity (Fig. 3A-F). On the day 1 after the surgery, HGF immunoreactivity was not observed in the stroma (Fig. 3A). Macrophages showed strong, and fibroblasts and endothelial cells revealed moderate HGF immunoreactivity on day 3 to day 14 after surgery (Fig. 3B-F). Faint c-Met immunoreactivity was present nonuniformly in the normal epithelium of the skin and colon. In contrast, regenerated colonic epithelium uniformly showed moderate c-Met immunoreactivity on day 3 (Fig. 4B) to day 14 (Fig. D). Colonic ductal cells next to regenerated colonic epithelium exhibited faint c-Met immunoreactivity in the basal cells (Fig. 4C, arrows). Regenerated skin epithelium of the prickle cell layer revealed strong c-Met immunoreactivity on day 3 (Fig. 4 A) to day 14 (Fig. 4D, E).

Competitive RT-PCR: To quantify the expression level of HGF and c-Met mRNA at each stage of healing of the artificial anus, competitive RT-PCR was employed. The HGF mRNA level increased gradually from day 1 to day 14 after the operation. On day 1, the concentration of HGF mRNA was about 0.001 attomole/ml, which increased up to 0.05 attomole/ml on day 14 (Fig. 5A, B). On the other hand, the c-Met mRNA level showed two peaks, on day 1 and day 7. The highest

concentration of c-Met mRNA was 0.005 attomole/ml, on day 1 (Fig. 5C, D).

In situ hybridization: To identify cells that overexpressed HGF or c-Met mRNA, ISH was performed. In normal colonic and skin tissue, faint expression of HGF mRNA was focally recognized in fibroblasts and macrophages (data not shown). HGF mRNA was overexpressed in fibroblasts (Fig. 6A, B, arrowheads), endothelial cells and macrophages (Fig. 6C, arrows) on day 14 after surgery, whereas colonic ductal cells and squamous epithelial cells did not express HGF mRNA. C-Met mRNA was weakly expressed in the normal epithelial cells of the skin and colon. In the artificial anus, c-Met mRNA was expressed strongly in basal to parabasal cells in the squamous epithelium of the skin (Fig. 6D, F) and moderately in the regenerated ductal epithelium of the colon on day 1 (Fig. 6D, G). Colonic ductal cells next to regenerated colonic epithelium expressed c-Met mRNA in a few basal cells (Fig. 6H, arrows). In situ hybridization used sense probe did not show any positive signals (Fig. 6E).

Discussion

HGF, also called the scatter factor, is a pleiotropic growth factor, originally identified as a mitogen for hepatocytes[1]. It is a kringle-containing polypeptide growth factor possessing structural homology with plasminogen[14]. In wound healing on various tissues, HGF has many roles, including in cell proliferation, cell motility, tissue organization, formation of duct-like structures and neovascularization [15,16]. HGF is synthesized as pro-HGF and then digested by a proteolytic enzyme named HGF activator[24]. Mature HGF is a heterodimeric protein consisting of a and b chains held together by a disulfide bond[17]. Only the mature HGF has biological activity and its effects are dependent on the presence of the highly specific HGF receptor (c-Met, product of the c-Met proto-oncogene) on the target cell membrane[11,12].

Clinically, construction of the artificial anus is used mainly in the management of anorectal diseases, such as anal-rectal carcinoma. In this study, we selected artificial anus in the rat as a unique wound healing model, because the artificial anal wound includes two different types of epithelium (the squamous epithelium of the skin and ductal epithelium of the colon) and some mesenchymal cells (fibroblasts, macrophages and endothelial cells) in the granulation tissue. The epithelial cells are considered to be the target cells of HGF, and the mesenchymal cells are reported to mainly synthesize HGF[8, 9, 10]. The model used in this study may also clarify the predominant target epithelium of HGF on the regenerative condition in vivo.

At the early stage of healing, squamous and ductal cell proliferation was recognized; however, fibroblast and endothelial cell proliferation was not observed yet. The epithelial cell proliferation was observed earlier than the formation of granulation tissue at the site of anastomosis. Mature HGF protein and its mRNA were detected by Western blotting and competitive RT-PCR, respectively. Moderate HGF immunoreactivity was recognized in regenerated epithelial cells in the early stage. This immunoreactivity in the epithelial cells was apparently not due to the protein being synthesized in these cells, because HGF mRNA expression was not recognized by ISH. The HGF is considered to be produced in other organs or tissues and then to bind to the c-Met receptor on the epithelial cells. Similar immunohistochemical staining results were reported previously for HGF[18]. In contrast, the increase in the c-Met mRNA level at the early healing stage may indicate that increased HGF/c-Met binding signals induce c-Met protein synthesis in the regenerated epithelial cells. HGF is reported to be secreted as a single chain of pro-HGF and stored in stromal compartments including type I, III, V and VI collagen in various tissues[19]. It is suggested that the stromal HGF or HGF in blood may take part in the stimulation of the epithelial cell growth during the early stage of wound healing in an endocrine or paracrine manner.

HGF has morphogenic potential that makes this molecule unique, in that it induces branching and formation of ductal epithelium in the late phase of wound healing[20,21]. In the rat gastric ulcer model, both HGF and c-Met expressions were more prominent on day 3 and day 15 of

healing of the gastric ulcer as determined by immunohistochemical analysis.[22]. In our studies, the HGF mRNA level was low at the early healing phase in rat artificial anus (day 1 and day 3) and then increased during the late phase (day 7 and day 14). In contrast, the c-Met mRNA level exhibited two peaks, the first during the early phase (day1) and the second during the late phase (day 7) of healing. C-Met and its mRNA were colocalized in regenerated ductal cells close to the anastomotic site. Near the anastomotic site, c-Met mRNA was overexpressed in the entire layer of ductal epithelium of the colon. In contrast, in the nonproliferative area away from the site of anastomosis, c-Met protein and its mRNA were localized only in the basal layer of the ductal epithelium. We also found the formation of normal ductal-like structures of regenerated epithelial cells during the late phase (day 7) of healing. It is suggested that the activation of the HGF/c-Met signaling pathway during the late stage of healing may be involved in not only cellular proliferation of the skin and colonic epithelium, but also in formation of ductal structure of colon on rat artificial anus. Increased HGF production in the artificial anus may be induced by cytokines such as interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α), reported previously as being inducers of HGF mRNA expression [23,24]. C-Met has a multifunctional docking site, binding Grb 2, Gab 1, PI 3 kinase and PLC-g, and these transduction signals may be involved in different functions, such as cell proliferation or tissue organization[25].

Interestingly, during process of wound healing in the rat artificial anus, HGF and c-Met showed differences in expression levels between the squamous cells of the skin and the colonic ductal cells. In our study, throughout the healing process of the artificial anus, HGF immunoreactivity was stronger in the squamous epithelium of the skin than in the ductal epithelium of the colon in the proliferative area. Furthermore expression of c-Met and its mRNA was more prominent in the squamous epithelium of the skin. We can suggest that these differences in the expression levels of HGF and c-Met between squamous cells and ductal cells may account for the differences in the effects on these epithelium.

In summary, HGF and c-Met were overexpressed in rat artificial anus, and HGF/c-Met may play a role in epithelial cell proliferation and formation of colonic ducts. HGF is suggested as having more predominant effects on the regenerating squamous cells in the skin of the artificial anus.

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