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Effect of hepatocyte growth factor on the expression of E- and P-cadherin in gastric carcinoma cell lines

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Abstract Hepatocyte growth factor (HGF), identical to scatter factor, (SF) is a secretory glycoprotein from fibroblasts which dissociates and increases the motility of various types of epithelial cells. After treatment of three gastric carcinoma cell lines (MKN-28, MKN-45 and TMK-1) with HGF (10 ng/ml), TMK-1 cells lost their tight cell to cell contact and showed marked scattering, while the two other cell lines remained unaffected. To learn about the underlying mechanism of the HGF induced scattering, we examined the expression of adhesion molecules and growth factor/receptor systems at the mRNA and protein level. The observed scattering of treated TMK-1 cells was associated with a reduction in the expression of E- and P-cadherin protein. The respective mRNA levels remained unchanged after HGF/SF treatment. In the two other cell lines, which showed no scattering, there were no changes in the expression of E- and P-cadherin. All other growth factors and their receptors examined (TGF-α, EGFR, c-*met* and c-*erb*B_2) remained constant and were not affected by HGF treatment. The results suggest that HGF/SF may regulate cell adhesion in gastric carcinomas via E-and P-cadherin expression at the protein level.

Key words Hepatocyte growth factor · Gastric cancer
Adhesion molecules · Cancer-stromal interactions · c-*met*

Introduction

Hepatocyte growth factor (HGF), identical to scatter factor (SF), is a secretory glycoprotein of stromal fibroblasts which enhances dissociation and motility of epithelial cells [17, 30, 33]. It has been recently reported that HGF not only induces morphological changes of cells, but also promotes their invasiveness and tumour progression [9, 24]. The cellular receptor for HGF has been determined to be c-*met* [4], a proto-oncogene which is over-expressed in various tumours [6, 7] and frequently amplified in gastric carcinomas [13].

Cadherins are a multigene family of transmembrane glycoproteins, which are located on the cell surface and responsible for calcium-dependent intercellular adhesion [29]. E-cadherin is expressed by almost all epithelial cells and is considered to be the main cadherin type for intercellular adhesion. P-cadherin was originally identified in mouse placental tissue as a molecule which appeared to act as a connector between the embryo and uterus [25]. As reported previously, down regulation of cadherins occurs during tumorigenesis and reversible loss of these molecules or alteration in function leads to changes in the cellular appearance and invasiveness of the tumour cells [3, 8, 18, 32]. An inverse correlation between expression of E-cadherin and lymph node metastasis has been reported for squamous cell carcinomas of the head and neck [23], breast carcinoma tissue [20] and prostate cancer [31].

In gastric carcinoma, E- and P-cadherin are reduced in dedifferentiated tumours and also in more advanced stages [15, 34]. Mutations of exons containing the calcium binding domain of the E-cadherin gene has been described by Becker et al. [2].

In this study, we investigated the effects of HGF on the expression of adhesion molecules as well as growth factors and their receptors in gastric carcinoma cell lines with different grades of differentiation.

Materials and methods

Two gastric carcinoma cell lines of the MKN series (MKN-28 derived from well-differentiated type of human gastric adenocarcinoma, and MKN-45 from poorly differentiated adenocarcinoma) were kindly provided by Dr. Suzuki (Fukushima Medical Univer-

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sity, Fukushima). TMK-1 was established from poorly differentiated adenocarcinoma in our laboratory [19].

These cell lines were routinely maintained in RPMI 1640 (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Whittaker, Walkersville, Ma., USA) under conditions of humidified 5% carbon dioxide in air at 37° C.

Cell culture were either performed on plastic dishes or on type I-collagen coated dishes (Corning, New York, USA).

Cells were grown to subconfluence in the above medium. After pre-incubation for 24 h in serum free medium, HGF in a concentration of 10 ng/ml was added. Cell harvesting was performed after 0, 1, 3, 12 and 24 h unless indicated otherwise. If cell culturing was performed on collagen coated dishes, we used the same conditions also for treated and untreated cells.

RNA was extracted by the guanidine isothiocyanate/caesium chloride method [22] Poly-A selected RNA (10 µg) was electrophoresed on 1.0% agarose-formaldehyde gel and blotted onto Zeta-Probe nylon filter membrane (Bio-Rad Laboratories). Hybridization and washing were performed as described previously [36]. The following c-DNA probes were used: (1) The 1.6 kb p-*met*H DNA probe was provided by the Japanese Cancer Research Resources Bank (JCRB) [21] (2) The 2.4 kb *Cla*I-*Cla*I fragment of EGFR c-DNA from pE7 by JCRB; (3) p CER 204, a 1.6 kb pair *Eco*RI-*Eco*RI fragment of *erb*B_2 gene was kindly provided by Dr. Y. Yamamoto; (4) 1.4 kb human TGF-α c-DNA was kindly provided by Dr. R. Derynck; (5) E- and P-cadherin c-DNA probe were kindly provided by Dr. S. Hirohashi [26] and (6) beta-actin probe was purchased from Oncor, Gaithersburg, M.D., USA

The filters were autoradiographed on Kodak XAR-5 films at – 80° C.

Western blot analysis was carried out as described previously [12]. The cells were lysed in lysis buffer (50 mM TRIS-HCl pH 7.4, 125 mM sodium chloride 0.1% (v/v) NP-40, 5 mM EDTA, 50 mM sodium fluoride, 50 µg/ml phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin (Sigma, St. Louis, Mo.), 10 µg/ml soybean trypsin inhibitor (Sigma), and 1 µg/ml aprotinin) and the cleared lysates containing 50 µg of protein were applied to a 7.5% SDS-polyacrylamide gel electrophoresis, followed by electrotransfer onto nitrocellulose filter (Schleicher and Schuell, Dassel, Germany). Anti-E-cadherin (HECD-1) and anti-P-cadherin (NCC-CAD-299) were kindly provided by Dr. Hirohashi (National Cancer Institute, Tokyo).

The establishment and specificity of HECD-1 and NCC-CAD-299 have been described by Shimoyama et al. [25] HECD-1 as a monoclonal antibody is capable of inducing the disruption of cell-cell adhesion in monolayer cultures of MCF-7 cells [20]. Anti- c-*met* polyclonal antibody, corresponding to amino acid position 1390–1408 of c-*met* product, was produced in our laboratory and anti-EGFR monoclonal antibody was obtained from Oncogene Science, Manhasset, N.Y. Anti-mouse IgG rabbit antibody (Tago, Burlingame, Calif., USA) were used in a secondary reaction. For detection of the immune complex, ECL Western-blotting detection system (Amersham, Aylesbury, UK) were used.

Indirect immunofluorescence analysis was performed on cells grown on multi-chamber slides (Nunc. Naperville, III., USA) either treated with HGF (10 ng/ml) or not. When treated cells showed scattering, the cells were fixed in ice-cold acetone. Slides were incubated with mouse monoclonal anti-E-cadherin antibody at 1/200 dilution, washed and reacted with an fluorescein isothiocyanate coniugated anti-rabbit secondary antibody (MBL) at 1/50 dilution. After glycerol mounting the slides were examined at 495 nm using a Nikon-microscope.

Treated and untreated TMK-1 cells (HGF in various concentrations [0, 1, 10 and 50 ng/ml] were pulse-labelled with tritiated-thymidine for 3 h. Radioactivity was determined by solubilizing cells with 1 M hydrochloric acid and counted in a liquid scintillation counter.

All experiments were repeated three times.

Results

To examine the morphological effect of HGF, TMK-1, MKN-28 and MKN-45 cells were treated with HGF in a concentration of 10 ng/ml. After 72 h of treatment, TMK-1 cells, which normally grow in tight, cluster like cell formations, showed a different morphological appearance. The cells lost their tight cell to cell contact and scattered throughout the culture dish. The cell shape changed from cuboidal to spindle form (Fig. 1). In contrast, MKN-28 and MKN-45 cells retained their morphological appearance and showed no scattering after treatment with various concentrations of HGF (5, 10, 15 and 20 ng/ml). These morphological changes were only obtained when we used culture dishes with a collagen coated surface; in cells cultured on plastic dishes no differences in appearance could be observed.

The cell number of TMK-1, MKN-28 and MKN-45 cells, counted on the fifth day, showed a slight increase in every case. However, this increase was not significant. No mitogenic effect was found after culturing on collagen coated dishes.

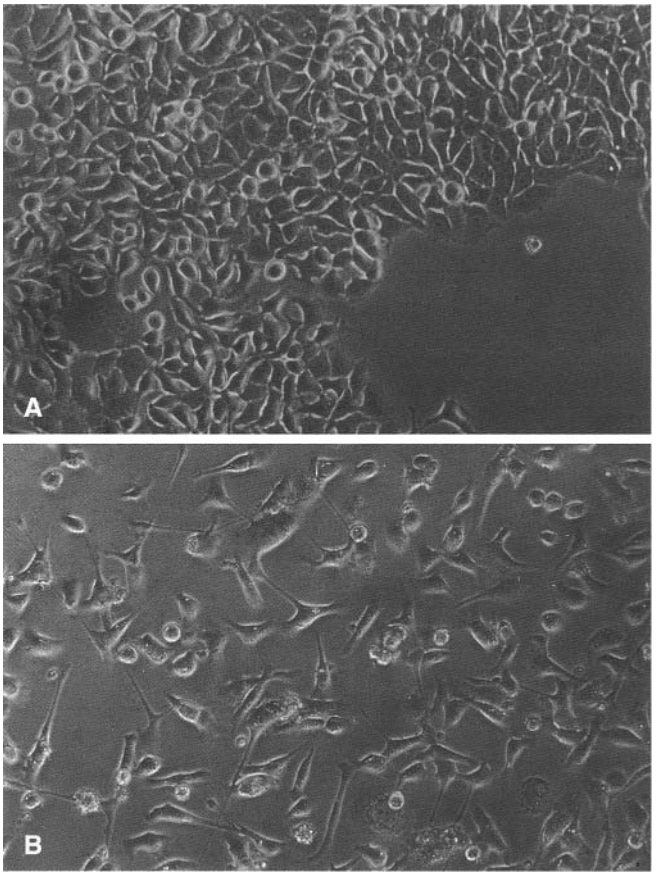

Fig. 1 Scattering effect of HGF on TMK-1 cells. TMK-1 cells were plated at a density of 5×10^4 cells/35 mm collagen coated dish and cultured in the absence (**A**) or presence (**B**) of HGF (10 ng/ml). **A** TMK-1 cells without HGF treatment grew in small cluster like cell formations with tight cell to cell contact. **B** TMK-1 cells after HGF treatment (72 hours). Cells showed scattering: they lost their tight cell to cell contact and the cell shape changed from cubic to spindle form

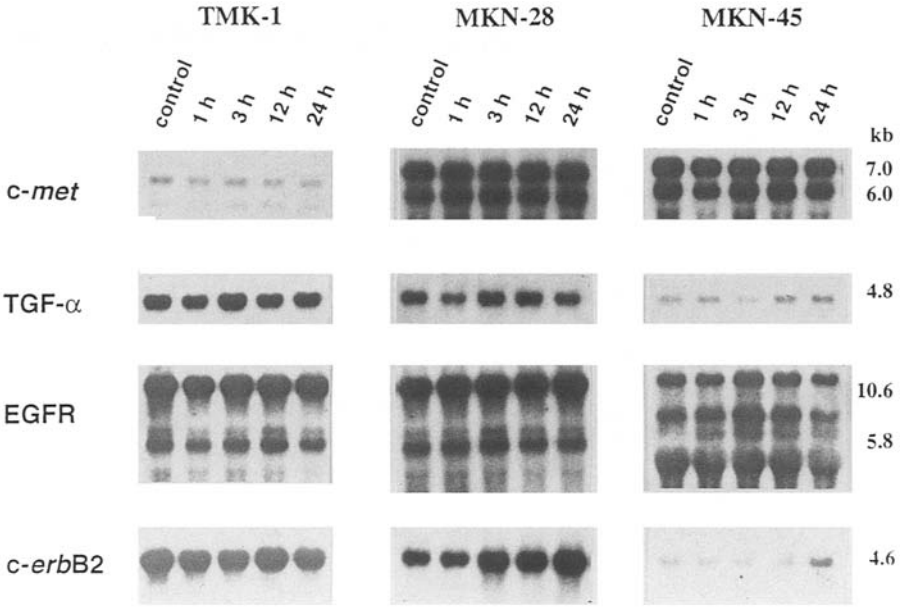

Fig. 2 Effect of HGF on the expression of various growth factor/receptor systems. Ten micrograms RNA of HGF treated cells were subjected to Northern blot analysis and probed with various cDNA probes. c-*met* and TGF-α mRNA expression were not affected by HGF treatment. In MKN-45 cells, EGFRmRNA expression was slightly induced after HGF treatment as well as c-*erb*B_2 mRNA in MKN-28 cells

To study possible changes in mRNA expression of various growth factors and their receptors after HGF treatment, the three cell lines (TMK-1, MKN-28 and MKN-45) were used. The levels of expression were determined by densitometry and the level of autoradiographic signal at each time was compared to that in the case without treatment. As shown in Fig. 2, HGF treatment did not affect the expression of c-*met* in all cell lines examined. EGFR mRNA was slightly induced after 3 h of HGF treatment in MKN-45 cells, but not in MKN-28 and TMK-1 cells. Induction was confirmed on the protein level (data not shown). The expression of c-*erb*B_2 was slightly induced in MKN-28 cells. TGF-α mRNA remained unchanged after HGF treatment. The levels of *FOS, MYC* and type IV Collagenase mRNA were not affected by HGF treatment of each cell line examined (data not shown).

HGF treatment did not affect the level E- and P-cadherin mRNA after culturing of the cell lines on plastic dishes over 3, 6, 12, 24, 48 or 72 h (Fig. 3A). Additional culturing on collagen coated dishes and treatment for a period of 24 h did also not change the mRNA levels of E- and P-cadherin also (Fig. 3B).

To determine whether the observed scattering in TMK-1 cells is due to loss of E- and P-cadherin protein, TMK-1 cells, grown on collagen coated dishes, were treated with HGF (10 ng/ml) over a longer period of time until the cells scattered.

As shown in Fig. 4, in TMK-1 cells, both E- and P-cadherin decreased with HGF treatment as compared with untreated cells. In case of P-cadherin, no protein was detectable after a treatment period of 72 h. c-*erb*B_2, c-*met* and EGFR levels in TMK-1 did not change after 96 h treatment with HGF.

The other cell lines examined showed no change in E- and P-cadherin with HGF treatment.

We next examined the changes in E- and P-cadherin protein distribution within cells through indirect immu-

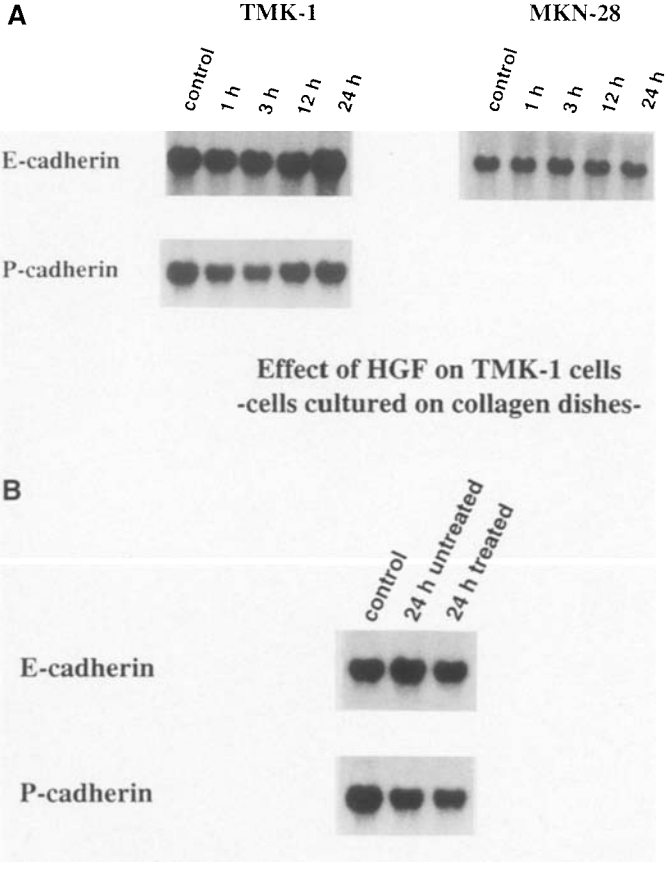

Fig. 3 Effect of HGF on the expression of E- and P-cadherin mRNA in TMK-1 and MKN-28 cells. E- and P-cadherin mRNA expression remained unchanged after HGF treatment in TMK-1 and MKN-28 cells using plastic and collagen coated dishes for culturing. **A** Cells were cultured on normal plastic dishes. **B** Cells were cultured on collagen coated dishes

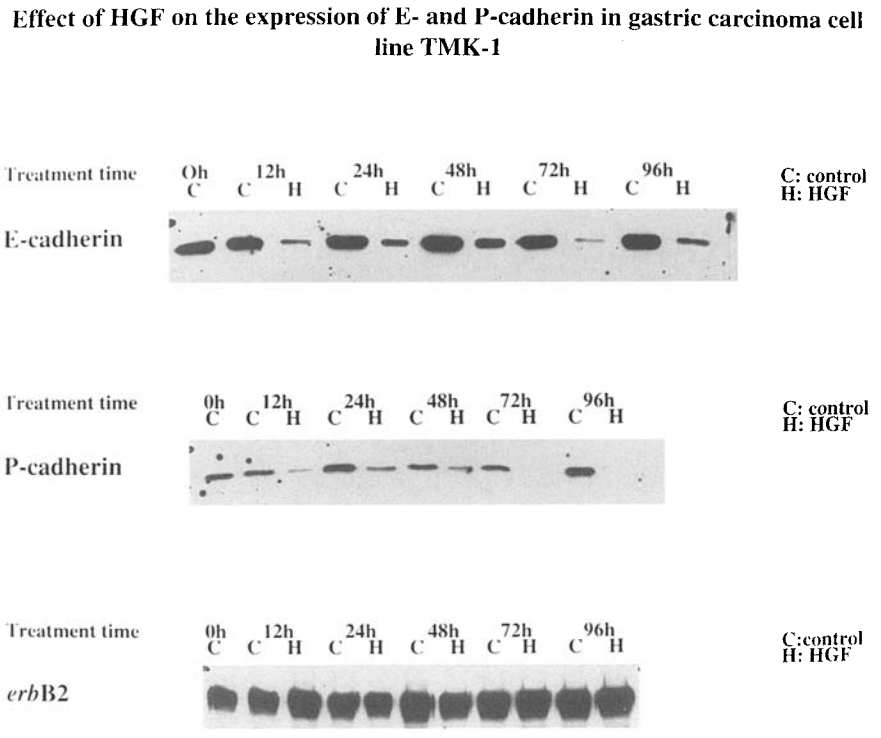

Fig. 4 Effect of HGF on the expression of E- and P-cadherin protein in TMK-1 cells. Cells were cultured on collagen coated dishes and treated as indicated. 50 µg protein lysate was subjected to Western blot analysis and probed with antibodies against E- and P-cadherin. Panel with anti-*erb*B2 was used for internal lysate control. In TMK-1 cells, E- and P-cadherin protein expression decreased with HGF treatment (treated cells indicated with "H" for HGF) as compared with untreated cells (indicated with "C" for control). P-cadherin was not detectable after a treatment period of 72 h

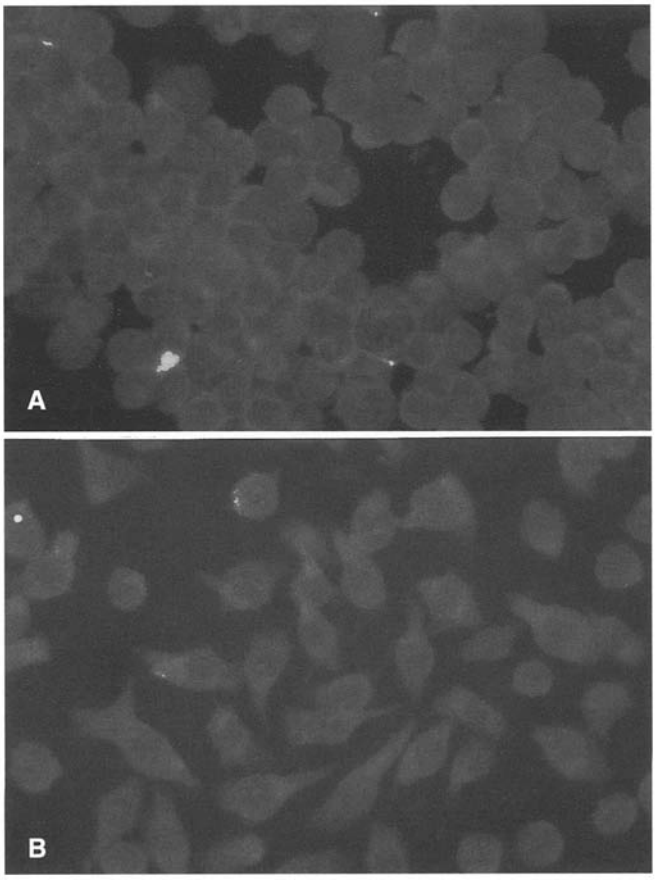

Fig. 5 Effects of HGF treatment on the expression of E-cadherin in TMK-1 cells. TMK-1 cells were plated at a density of 5×10^4 cells/35 mm (collagen coated) dish in the absence (**A**) or presence (**B**) of HGF [10 ng/ml]. After 72 h, immunofluorescence analysis for E-cadherin was performed using fluorescein isothiocyanate conjugated antibodies. **A** Untreated TMK-1 cells showed a strong immunoreactivity against E- and P-cadherin along the plasmalemma. **B** TMK-1 cells treated with HGF. The cells showed a weaker immunoreactivity which was not specifically localised to the plasmalemma

nofluorescence analysis before and after HGF treatment of TMK-1 and MKN-28 cells. Untreated TMK-1 cells showed strong immunoreactivity against E- and P-cadherin along the plasmalemma (Fig. 5A), whereas in HGF-treated, scattered cells the immunoreactivity was weaker and not specifically localized to the cell border (Fig. 5B). MKN-28 cells, which did not scatter in response to HGF, revealed unchanged distribution of E- and P-cadherin after the treatment (data not shown).

Discussion

We have shown that HGF caused morphological changes in the gastric carcinoma cell line TMK-1, established from a poorly differentiated gastric carcinoma. Treated cells lost their tight cell to cell contact and scattered on collagen gel. Two other cell lines originating from well-differentiated gastric carcinomas, remained unchanged after HGF treatment. To elucidate the underlying mechanisms of the different response to HGF, we examined the expression of E- and P-cadherin as well as various growth factors and their receptors. Only in case of the two adhesion molecules E- and P-cadherin, differences in expression after HGF treatment could be detected at the protein level, whereas the tested growth factors and their correspondent receptors remained unchanged on RNA as well as at the protein level.

Using an indirect immunofluorescence technique, we found in agreemet with the results of Albelda et al. [1] and Mayer et al. [15] the transmembrane adhesion molecules E- and P-cadherin were expressed predominantly at the lateral cell border of the tumour cells. After HGF treatment, there was a reduction of the immunoreactivity on the cell membrane of the scattered cells, suggesting a redistribution of E-/P-cadherin from the cell border to regions inside the cell body. Why are the observed changes of these two molecules only seen at the protein but not the mRNA level? Several explanations are possible. As the amount of mRNA remained stable under HGF influence, post-transcriptional effects may have occurred, leading to structurally modified E- and P-cadherin, which are then unable to function as adhesion molecules. If this hypothesis is correct, tumour surrounding fibroblasts might influence the adhesion properties of the malignant cells and also their differentiation via HGF. Tahara et al. [27] proposed that the HGF secretion from fibroblasts could be stimulated by TGF-α, TGF-β and interleukins (especially IL-1α), which are produced by tumour cells as a circuit. A second possibility could be a transient down regulation of cadherin instead of a irreversible loss of E-cadherin expression. This was described by Vleminckx et al. [32], who found that tumour cells are able to re-express E-cadherin. Mareel et al. [14] found also a reversible down-regulation of E-cadherin and reported an uneven expression within the same tumour: differentiated parts expressed high amounts, while undifferentiated areas showed a reduced expression. [14] They argued, that this down regulation is due to local host factors, for instance growth factors. Another possibility is that HGF may enhance-the turnover of the two adhesion molecules, without affecting their production or molecular structure.

During embryonic development, Takeichi et al. reported that E-cadherin expression is down regulated and that this is associated with a transition from epithelial to fibroblastic morphotype of the embryonic cells. The reverse transition is accompanied by up-regulation of E-cadherin [28]. Commonly, the fibroblastic like cells seemed to be more malignant, representing an invasive phenotype [3]. The HGF induced morphological effects (scattering) could be interpreted as an example of a more invasive phenotype as a result of a reversible downregulation or conformational alteration of E- and P-cadherin. Interestingly, the above described E- and P-cadherin decrease were only observed on cells which were cultured and treated on plastic dishes coated with a collagen covered surface. The importance of epithelial – matrix interactions was reported recently by several other studies [5, 10, 24], especially on MDCK cells which formed branching, kidney tubule reminiscent structures after HGF treatment [16]. It is highly possible that HGF induced scattering requires defined epithelial – matrix interactions as also reported for other biological functions of this cytokine [5, 9, 11].

Taking into account that E- and P-cadherin were both expressed in gastric carcinomas [15, 23, 34] and are closely related to cellular dedifferentiation and tumour progression, it is reasonable to assume that HGF may be linked to the development of a poorly differentiated type of stomach cancer, affecting the molecular structure (post-transcriptional) or the amount (enhanced turnover) of cadherins.

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