Stimulation of Urokinase-type Plasminogen Activator Expression by Blockage of E-Cadherin-dependent Cell-Cell Adhesion

Uwe H. Frixen and Yoshikuni Nagamine

ABSTRACT

Decreased expression of the cell-cell adhesion molecule, E-cadherin, promotes dedifferentiation and invasiveness of human carcinoma cells, whereas this process can be reversed by reexpression of E-cadherin (U. H. Frixen et al., J. Cell Biol., 113: 173–185, 1991; J. H. Schipper et al., Cancer Res., 51: 6228–6237, 1991). In this work we studied the involvement of extracellular matrix-degrading proteases in E-cadherin-dependent tumor cell invasion. When T47D and MCF-7 human differentiated breast carcinoma cells were treated with the E-cadherin antibody DECMA (decom-pacting monoclonal antibody) the cells dissociated from each other and lost their epithelialoid morphology, paralleled with a rise in the secretion of urokinase-type plasminogen activator (uPA) into the extracellular milieu as determined by zymography. The stimulation of uPA required protein synthesis. Furthermore, when DECMA-treated T47D cells were cultured on artificial collagen matrices the induced invasiveness correlated with accumulation of uPA in the culture medium, and uPA antibodies inhibited this invasion process. Actin filaments which are thought to be associated with the cytoplasmic part of E-cadherin were disrupted after treatment of T47D cells with DECMA. These results suggest a link between cell-cell adhesion, the integrity of actin filaments, and the regulation of uPA biosynthesis.

INTRODUCTION

Recent studies on the invasiveness of epithelial tumor cells have shown an important role for E-cadherin, an epithelium-specific transmembrane cell-cell adhesive molecule. Expression of E-cadherin is inversely correlated with dedifferentiation and invasiveness of human carcinomas and epithelial tumor cell lines from other mammalian sources (1–3). The molecule is causally responsible for the regulation of invasiveness in vitro because transfection of an E-cadherin expression vector prevents invasion, whereas transfection of an antisense RNA expression vector promotes invasion (1, 4). Treatment of cells with anti-Arc-1 or DECMA2 antibodies against E-cadherin caused dissociation and induced a fibroblastoid morphology and invasiveness of MDCK canine renal epithelial (3) and human differentiated breast carcinoma cell lines (1). However, the loss of E-cadherin-mediated cell-cell adhesion can only be regarded as an initiating step in the whole invasion process. There must follow the degradation of extracellular matrix that facilitates tumor cell infiltration. Several laboratories have already found that expression of matrix-degrading enzymes such as collagenase, tissue-type plasminogen activator and uPA are directly correlated with invasiveness and metastatic potential of various mammalian cells. Among these proteolytic enzymes, uPA has been reported to play a major role in a wide spectrum of tumor types including carcinomas (Refs. 5–8; for uPA review, see Ref. 9).

Because E-cadherin influences the invasive capacity of carcinoma cells, we considered the possibility that it mediates regulation of uPA production in these cells. Evidence for a possible association between E-cadherin and uPA is provided by uPA gene regulation through the cytostkeleton; i.e., the disruption of actin filaments by cytochalasin induces uPA gene expression in porcine kidney epithelial cells (10). The cortical actin bundles which colocalize with E-cadherin (11) are likely to be disorganized during the morphological changes induced by E-cadherin antibody. Thus, we hypothesized that uPA could be stimulated through treatment of differentiated, noninvasive human carcinoma cells with cell-dissociating and invasion-inducing E-cadherin antibodies. We utilized antibody DECMA which had been generated against mouse uvomorulin (E-cadherin) (12) and which we found to cross-react with human T47D and MCF-7 breast carcinoma cells. The organization of actin filaments dependent on E-cadherin function was also analyzed.

MATERIALS AND METHODS

Cell Culture, Antibodies, and Reagents. T47D and MCF-7 cell lines originating from human differentiated breast carcinomas were provided from N. Hynes and B. Hemmings, respectively (Basel, Switzerland), and maintained in DMEM containing 10% fetal calf serum. A rat hybridoma cell line producing DECMA-1 (12), in this article referred to as DECMA, was generously provided by D. Vestweber and R. Kemler (Freeburg, Germany). Rat ascitic fluid containing DECMA was purchased from Sigma Chemical Co., St. Louis, MO. Anti-Arc-1 mouse monoclonal antibody was obtained from J. Behrens, Essen, Germany. Rabbit antisera to human uPA were kindly provided by T-C. Wun, Monsanto Co., Ltd., and W-D. Schleuning, Schering AG, Berlin, Germany. Human plasminogen was purchased from Kabl Vitrum, Stockholm, Sweden. Cycloheximide and actinomycin D were obtained from Sigma and used at concentrations of 10 and 2 µg/ml, respectively.

Cell Dissociation by DECMA and Invasion Assays. Unless otherwise indicated, DECMA was used as hybridoma supernatant dialyzed against DMEM (M, cutoff, 1.2 x 10⁶; the dialysis reservoir was used for control treatments). Cells (2.5 x 10⁶) suspended in 300 µl DMEM-containing or control culture medium were seeded into 24-well plates. Cell culture supernatants were collected at the times indicated, centrifuged, and subjected to nonreducing sodium dodecylsulfate-polyacrylamide gel electrophoresis after normalization of the applied volumes to the cell numbers. Occasionally, other preparations of DECMA were used including rat ascitic fluid (dialyzed against PBS and diluted 1:300) and DECMA IgG affinity purified from hybridoma supernatants using an anti-rat IgG-Affigel 10 column (Pharmacia). The specific IgG was eluted with 0.1  l glycine-0.1  l NaCl, pH 2.5, immediately neutralized, and dialyzed against PBS.

The collagen invasion assay was performed as described (3). T47D cells, 1.2 x 10⁵, suspended in 1 ml of culture medium containing DECMA or the appropriate control were seeded on top of the collagen gel in a well of a 6-well plate. uPA antisera (1:100) was added to the culture medium 1 h after the addition of DECMA.

Fluorescence Microscopy. For E-cadherin staining, T47D and MCF-7 cells grown on glass coverslips were fixed for 30 min with 3% formaldehyde at room temperature, permeabilized with 1% Triton X-100, and incubated with DECMA hybridoma supernatant followed by fluorescein-labeled anti-rat IgG. To stain actin filaments, T47D cells were fixed and permeabilized as above and incubated with rhodamine-labeled phalloidin. Fluorescent cells were viewed in an Axiophot microscope (Zeiss, Germany).

Zymographic Assays. Five to 10 µl of medium from untreated and treated cells was subjected to 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis under nonreducing conditions. The polyacrylamide gel was overlaid with a gel containing 1.3% skimmed dry milk–40  µg/ml human plasminogen in 0.8% agarose. After 1 day of incubation at 37°C, plasminogen activator activity was detected as bands of proteolytic clearing in the agarose gel (13).
Cell-associated PA was detected by overlaying cell monolayers with 0.5% agarose containing 2% skimmed dry milk, 20 μg/ml plasminogen, and DMEM as described (14).

RESULTS

To study the effect of E-cadherin dysfunction on uPA expression, human differentiated breast carcinoma cell lines were treated with E-cadherin antibody. Because monoclonal antibodies previously generated against the human molecule (1) did not dissociate human epithelioid cells (data not shown), DECMA was used in this study. This antibody, which had been raised against mouse E-cadherin (12), also recognized the human counterpart. T47D breast carcinoma cells which are epithelioid in morphology (Fig. 1a) were completely dissociated from each other after 24 h treatment with DECMA (Fig. 1b). Immunofluorescence localization studies indicated that the antibody accumulated around T47D cells at sites of cell-cell contact (Fig. 1c).

Conditioned medium of these cells cultured in the presence or absence of DECMA for different times was subjected to zymography. Cells released a PA of M₉ 43,000 into the culture medium. The level of enzyme activity was significantly enhanced in the presence of DECMA (Fig. 2A). Maximal enhancement was obtained after 25 h of treatment with DECMA. When uPA antiserum was included in the plasminogen-casein underlay, the appearance of the M₉ 43,000 band was inhibited, indicating that the PA is of the urokinase-type (Fig. 2B). When conditioned medium from MCF-7 cells was incubated for 25 or 50 h with DECMA and subjected to zymography, we observed increased levels of two PAs of M₉ 49,000 and M₉ 56,000 (Fig. 2A). The activity of the M₉ 49,000 protein was inhibited by the uPA antibodies, whereas activity of the M₉ 56,000 protein was not affected (Fig. 2B). This indicates that the M₉ 49,000 species is immunologically related to uPA. The difference of apparent molecular weights of uPA between T47D and MCF7 cells might reflect the different extent of protein glycosylation, which we have not investigated further in this study. The M₉ 56,000 species is presumably tissue-type plasminogen activator that also converts plasminogen into plasmin. In both cell lines, the extent of PA induction correlated with the extent of cell dissociation (data not shown). The decline of PA activity after 50 h with or without DECMA (Fig. 2A) is possibly due to nonspecific protein degradation or inactivation. The high molecular weight bands of M₉ 90,000–100,000 appearing after zymographic analysis of conditioned medium from T47D and MCF-7 cells most likely represent complexes of PA with its inhibitor (15) that were not further analyzed in this study.

Various types and preparations of E-cadherin antibodies were tested to confirm the correlation between uPA induction and anti-E-cadherin-mediated cell dissociation. After 25 h of treatment, conditioned medium was subjected to zymography. The highest induction of uPA was observed using DECMA hybridoma supernatant (Fig. 3, compare Lanes 3 and 4). DECMA obtained from Sigma (Lane 5) and DECMA IgG affinity-purified from hybridoma cell culture supernatants also significantly induced uPA (Lane 7). The control for the affinity-purified antibody (PBS used for dialysis of affinity column eluted antibody) (Lane 6) and anti Arc-1 monoclonal antibody (Lane 2) which had been raised against canine E-cadherin (16) and does not cross-react with the human T47D cells did not increase basal uPA levels. DECMA hybridoma supernatant by itself did not contain any uPA activity (Lane 1).

Fig. 1. Cell dissociation and reaction with the cell-to-cell membranes of human T47D differentiated carcinoma cells after incubation with monoclonal E-cadherin antibody DECMA. a and b, phase contrast appearance of the cells treated 25 h with control medium (a) and DECMA-containing medium (b); bar, 70 μm. c and d, immunofluorescence staining of cells, using DECMA as first antibody (c) or PBS instead of DECMA (d); bar, 30 μm.
LOSS OF E-CADHERIN FUNCTION STIMULATES uPA

Fig. 2. Stimulation of plasminogen activators upon loss of E-cadherin function in T47D and MCF-7 cells using DECMA. T47D and MCF-7 human carcinoma cells were cultured in the presence or absence of E-cadherin antibody DECMA for the times indicated and conditioned medium was subjected to zymography. The volume of medium analyzed was normalized for cell number. A, expression of a M, 43,000 protein in T47D cells and of a protein doublet of M, 56,000 and M, 49,000 in MCF-7 cells. B, inhibition of the appearance of the M, 43,000 and M, 49,000 proteins after inclusion of uPA antibodies in zymography, indicating that the M, 43,000 T47D and M, 49,000 MCF-7 proteins are of the uPA type. The higher molecular weight (M, 56,000) species of the MCF-7 cells is presumably tissue-type plasminogen activator.

Fig. 3. Stimulation of uPA after treatment of T47D cells with various E-cadherin antibodies. uPA from conditioned medium of T47D cells treated for 25 h with E-cadherin antibodies were analyzed by zymography. Lane 1, DECMA-containing hybridoma medium not conditioned by the T47D cells; Lanes 2-7, conditioned medium of T47D cells treated with: irrelevant ARC-1 antibody (see "Materials and Methods") (Lane 2); control medium (Lane 3); DECMA (Lane 4); DECMA purchased commercially (Sigma) (Lane 5); PBS used for dialysis of affinity column-eluted DECMA IgG (Lane 6); and affinity-purified DECMA IgG (10 μg/ml culture medium) (Lane 7).

The DECMA-mediated induction of uPA was dependent on protein and RNA synthesis. The increase in uPA reactivity in the culture medium of T47D cells (Fig. 4, Lane 2 versus 1) was abolished when cells had been cultured for 5 h with DECMA in the presence of cycloheximide or actinomycin D (Fig. 4, Lanes 4 and 6, respectively).

Because uPA was reported to have maximal matrix-degrading and invasion-inducing potential when bound to its cell surface receptor (for review, see Ref. 9), we analyzed cell-associated enzyme activity. In a cell overlay assay, we found a significant increase over basal level in the number and size of proteolytic plaques surrounding T47D cell colonies after 25 and 50 h of incubation with DECMA (Fig. 5). When DECMA-treated T47D cells were cultured on collagen gels, the invasion capacity of the cells and the content of uPA in the medium also increased. In 2 invasion experiments, the E-cadherin antibody significantly induced the infiltration of T47D cells into the collagen matrix from day 2 onward (for details, see Table 1). The presence of uPA antiserum in the culture medium inhibited the E-cadherin-dependent invasiveness by up to 78.1% (see Table 1, invasion experiment I, day 2). Zymographic analysis of conditioned medium collected every 24 h during the invasion experiment revealed a correlation between the extent of invasiveness and the level of uPA induction (compare Fig. 6 and Table 1). Moreover, the extent of uPA stimulation was in good agreement with the degree of invasion inhibition by the uPA antibodies.

Because the dissociation of the carcinoma cells by DECMA was accompanied by drastic morphological changes, we were interested in a possible involvement of the actin cytoskeleton which colocalizes with E-cadherin at cell-cell boundaries (11). T47D cells were treated for 25 h with DECMA to achieve full dissociation, and then the organization of actin filaments was analyzed by staining with rhodamine-phalloidin and visualized by fluorescence microscopy. While the microfilaments crossed the entire length of control cells, treatment of the cells with E-cadherin antibody resulted in the disruption of the network. A characteristic example is given in Fig. 7. The organization of microtubule stained with a tubulin antibody was found not to be altered in dissociated T47D and MCF-7 cells (data not shown).

Fig. 4. Dependency of uPA stimulation on protein and RNA synthesis. T47D cells were treated for 5 h with DECMA in the presence or absence of cycloheximide and actinomycin D. Odd numbers, control treatments; even numbers, DECMA treatments; Lanes 1 and 2, no inhibitors; Lanes 3 and 4, cycloheximide; Lanes 5 and 6, actinomycin D.
The values represent means of 12 analyzed microscopic view fields showing between 425 and 532 cells in the monolayer on day 3 of the experiment.

The monoclonal anti-E-cadherin antibody DECMA (used as hybridoma supernatant, commercially purchased ascitic fluid, or affinity-purified IgG) dissociated differentiated T47D and MCF-7 human breast carcinoma cells with concomitant stimulation of uPA activity in the medium. The most marked effect was observed with DECMA hybridoma supernatant. The lesser increase of uPA obtained with other DECMA preparations (ascitic fluid and purified IgG) is most likely due to partial inactivation of the antibodies because they were less effective in dissociating cells (data not shown). Anti-Arc-1 monoclonal antibody against canine E-cadherin, which did not cross-react with the T47D and MCF-7 cells, did not augment uPA activity. These data indicate that uPA is induced by a specific inhibition of E-cadherin function in these cells.

Expression of E-cadherin has been shown to be inversely related to invasiveness of tumor cells of epithelial origin (1–4). Accordingly, we showed here that T47D cells invade an artificial collagen matrix upon treatment with DECMA. Invasiveness is attributable, at least partly, to increased levels of uPA in the extracellular milieu because it was partially suppressed by specific antibodies against human uPA. The extent of invasiveness and of uPA stimulation due to the action of E-cadherin antibody as well as the partial prevention of invasion by uPA antibodies correlated well in the two experiments performed. Incomplete inhibition of invasiveness by the uPA antibodies may be due to the fact that some other proteases such as collagenase or stromelysin are also involved in matrix degradation in this system or that some fraction of uPA is inaccessible to the antibody or that amount of the antibody was not sufficient. Presently we cannot distinguish between these possibilities.

DISCUSSION

In this study we showed that the expression of uPA is up-regulated through the loss of E-cadherin-mediated cell-cell adhesion. The monoclonal anti-E-cadherin antibody DECMA (used as hybridoma supernatant, commercially purchased ascitic fluid, or affinity-purified IgG) dissociated differentiated T47D and MCF-7 human breast carcinoma cells with concomitant stimulation of uPA activity in the medium. The most marked effect was observed with DECMA hybridoma supernatant. The lesser increase of uPA obtained with other DECMA preparations (ascitic fluid and purified IgG) is most likely due to partial inactivation of the antibodies because they were less effective in dissociating cells (data not shown). Anti-Arc-1 monoclonal antibody against canine E-cadherin, which did not cross-react with the T47D and MCF-7 cells, did not augment uPA activity. These data indicate that uPA is induced by a specific inhibition of E-cadherin function in these cells.

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After 1 day of DECMA treatment of the carcinoma cells cultured on the collagen gel, only an insignificant rise in uPA activity was detected compared to the drastic effects at the same time point of treatment of the cells cultured in the plastic dish. This can be explained by tighter cell-substrate adhesion on top of the collagen gel which in fact led to a later onset of cell dissociation followed by infiltration on day 2 of the invasion experiment. Our results on the tumor cell invasion coincident with uPA stimulation triggered by the loss of E-cadherin function allow the following conclusions: (a) the reaction of the uPA antibodies is specific because they only reduce the invasive behavior when significant levels of uPA are present, and the higher the stimulation of uPA, the higher the degree of invasion inhibition; (b) uPA is the main protease to facilitate the invasion of T47D cells since uPA antibodies inhibit this process by nearly 80%, but other proteolytic enzymes may also be involved (see references in Ref. 17); and (c) the stimulation of uPA detected during the invasion experiments confirms the involvement of this protease in malignancy of breast and other carcinomas as reported from various laboratories (5–8).

Ossowski et al. (18) reported on the importance of cell-associated uPA. Using a cell line expressing uPA and another cell-line expressing the uPA receptor, they demonstrated that the invasiveness was maximal when cell lines had been cocultured to permit ligand-receptor interaction, rendering uPA active at the cell surface. To address the question of uPA being bound to its cell surface receptor upon stimulation with E-cadherin antibody, we used an easy zymographic cell overlay technique. We found a drastic induction of T47D cell-associated proteolytic plaques, which we assume to be attributable to uPA because this is the only PA found in conditioned medium of these cells and because a cell surface receptor for tissue-type PA is not known. An interesting possibility would be a synergistic effect of the loss of E-cadherin function and induction of uPA receptor at the same time as the induction of uPA. Whether DECMA modulates the concentration of cell-surface uPA receptor remains to be seen.

**Table 1.** E-cadherin-dependent invasion of T47D breast carcinoma cells and its sensitivity to uPA antibodies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
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<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
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<tr>
<td>DECMA</td>
<td>4.0</td>
<td>3.2</td>
<td>7.2</td>
<td>n.d.</td>
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<tr>
<td>DECMA + Anti-uPA 1'</td>
<td>6.7</td>
<td>16.0</td>
<td>23.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DECMA + Anti-uPA 2'</td>
<td>4.9 (66.7)</td>
<td>9.4 (51.6)</td>
<td>16.3 (45.2)</td>
<td></td>
<td></td>
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<tr>
<td>DECMA + Anti-uPA 2</td>
<td>7.5 (~29.6)</td>
<td>6.0 (78.1)</td>
<td>14.9 (53.6)</td>
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<table>
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<th>Experiment II</th>
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<td>Control</td>
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<tr>
<td>DECMA</td>
<td>2.9</td>
<td>5.9</td>
<td>11.8</td>
<td>12.8</td>
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<tr>
<td>DECMA + Anti-uPA 2</td>
<td>15.8</td>
<td>15.2</td>
<td>40.5</td>
<td>24.6</td>
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<tr>
<td>DECMA + Anti-uPA 2</td>
<td>8.6 (55.8)</td>
<td>9.4 (62.4)</td>
<td>19.5 (73.2)</td>
<td>16.6 (67.8)</td>
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</tbody>
</table>

* n.d., not determined.

The percentage of invasion was determined by the number of cells which penetrated into the collagen gel related to the number of cells in the monolayer on top of the matrix. The values represent means of 12 analyzed microscopic view fields showing between 425 and 532 cells in the monolayer on day 3 of the experiment.

* Anti-human uPA antiserum (1:100) was added to the E-cadherin antibody-containing culture. In experiment I, two different batches of uPA antibodies were used.

* Numbers in parentheses, percentages of inhibition of DECMA-dependent invasion (calculated after subtraction of the control values).

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What are the mechanisms underlying the enhancement of uPA activity by the anti-E-cadherin antibody? The induction of uPA activity by DECMA was almost completely abolished by an inhibitor of protein synthesis, cycloheximide, and by an inhibitor of RNA synthesis, actinomycin D, indicating that the induction of uPA depends on de novo RNA synthesis, which was confirmed by a rise in uPA mRNA (data not shown).

The colocalization of E-cadherin and cortical actin bundles (11) together with the morphological changes of T47D cells treated with E-cadherin antibody prompted us to look into the organization of the actin cytoskeleton. We indeed found disruption of the microfilaments. It is possible that these alterations are due only to the morphological changes of cells per se, although the microtubule network was not affected (data not shown). The detailed molecular mechanism underlying the breakdown of the actin cytoskeleton is worth further study. In LLC-PK1 cells, a cell line derived from porcine renal epithelia, uPA expression is induced by the reorganization of tubulin cytoskeleton (through treatment with colchicine or nocodazole) or actin cytoskeleton (through treatment with cytochalasin B) (10). Induction is due to the transcriptional activation of the uPA gene and through a mechanism independent of protein kinase C and cyclic AMP-dependent protein kinase (10). The induction is not a nonspecific effect of cytoskeletal reorganization on nuclear function, because the induction is specific for the uPA gene (10), and is mediated by a specific cis-acting element in the uPA gene promoter. Because DECMA causes the reorganization of actin cytoskeleton, it is tempting to speculate that uPA expression induced by DECMA in T47D and MCF-7 cells is achieved through a similar mechanism as uPA induction by cytoskeletal reorganization in LLC-PK1 cells.

In this work we used specific antibodies to inactivate E-cadherin. The obvious question is whether there is a situation where a physiological signal causes the dynamic modulation of E-cadherin function. The function of E-cadherin is dependent on Ca^{2+} (19) and, therefore, if there is a signal that introduces changes in local Ca^{2+} concentration it may well be a regulator of E-cadherin function. So far, there is no evidence to suggest the presence of a regulator of extracellular Ca^{2+} concentration, although this does not exclude such a possibility. Another possibility is the positive feedback of uPA expression through E-cadherin. When proteolytic activity in the vicinity of the cell is enhanced through a pathway involving PA or other proteolytic enzymes, E-cadherin might also be subjected to proteolysis, which in turn would lead to uPA expression. Thus, E-cadherin would provide means to sustain or enhance proteolytic processes induced by other mechanisms.

To summarize, our work suggests that the loss of normal E-cadherin function followed by reorganization of the actin cytoskeleton directs a signaling pathway to up-regulate uPA expression leading to an increased level of proteolysis around the tumor cell to facilitate invasion. More detailed investigations need to be performed especially with respect to the transcriptional regulation of uPA in this system.

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