Loss of E-cadherin in the vicinity of necrosis in colorectal carcinomas: Association with NFκB expression

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Abstract. The transcription factor NFκB regulates the expression of several tumor-related molecules associated with tumor progression and metastasis. However, the precise mechanisms by which its activation mediates these processes in diverse tumors are unknown. In this study we determined the expression of NFκB in various colorectal carcinoma cell lines, in a series of 90 non-metastatic and metastatic colorectal tumors and in an in vitro 3D-spheroid model of HT-29 cells simulating morphological hallmark of these adenocarcinomas, namely neoplastic glandular nests around a necrotic center. We show that the inactive cytoplasmic NFκB form is evidently up-regulated in the tumor epithelium, especially in the metastatic cases, as compared to normal tissue. We found that in situ nuclear NFκB staining is characteristic for cells that are still viable but dissociated from the surrounding cohesive tumor tissue and destined to die. Evidence for a possible association between NFκB expression and loss of cell adhesion mediated by E-cadherin function has been provided in vivo and in vitro using the HT-29 3D-spheroid model. In both cases, we found a strong correlation between activation of NFκB and loss of E-cadherin expression. Considering the fact that cancer cell necrosis plays a crucial role in metastasis, NFκB activation mediated by loss of E-cadherin may represent an essential, even initial event in this process. Furthermore, we present in vitro data implicating LPS, the endotoxin of gram-negative bacteria, in the triggering of NFκB up-regulation. Thus, release of bacterial endotoxin may essentially contribute to the progression of colon cancer in vivo.

Introduction

The evolutionarily conserved NFκB (nuclear factor-κB) protein was originally discovered in B cells as a nuclear factor binding to the enhancer of the kappa light chain of immunoglobulin (1). Currently its expression in all cell types of diverse species from the fruit fly Drosophila to man has been established. NFκB belongs to the Rel/NFκB family encompassing NFκB1 (p50), NFκB2 (p52) and the Rel proteins RelA (p65), RelB and c-Rel (2-4). The classical NFκB occurs as a dimer consisting of two proteins, p50 and p65. In normal cells, except for proliferating T and B cells and thymocytes, monocytes and astrocytes, it forms an inactive complex with inhibitory proteins, members of the IκB (inhibitor of nuclear factor-κB) family. Already these features indicate association of NFκB with proliferative processes. In agreement with that it has been found that in most tumor cell lines, derived from both hematopoietic and solid tumors, NFκB is active and that its inappropriate activation mediates both tumorigenesis and inflammation.

The inactive IκB-bond NFκB form is retained in the cytoplasm. To translocate into the nucleus NFκB/IκB binding is released by rapid IκB phosphorylation and degradation via the ubiquitin-proteosome pathway. As is currently known the active NFκB induces the expression of a variety of genes regulating the immune response, growth, cell survival and apoptosis by binding to their specific kB-elements. Thus, proper development of cells depends on precise activation/inactivation of this transcriptional regulator.

As mentioned above, a constitutive activation of NFκB has been reported in nearly all tumor tissues including carcinomas of the gastrointestinal tract. Since the expression of oncogenes such as Ras and c-myc is mediated by NFκB, its constitutive activation has numerous consequences for tumor development.

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and progression. Furthermore, angiogenesis, tumor invasion and metastasis are regulated by numerous NFκB-regulated gene products such as matrix metalloproteinases, chemokines, growth factors and the adhesion molecules ICAM-1, VCAM-1 and E-selectin. In human prostate cancer cells inactivation of NFκB results in suppression of angiogenesis, invasion and metastasis (5) and in a murine lung alveolar carcinoma cell line, Line 1, in down-regulation of prometastatic factors and up-regulation of antimetastatic factors preventing intravasation of tumor cells (6). These data provide a strong indication that NFκB essentially contributes to cancer.

The past few years have brought tremendous progress in understanding the mechanisms of NFκB-modulated responses and their significance in tumor behaviour. However, the precise mechanisms by which NFκB activation mediates progression and metastasis of specific cancers still remain to be elucidated. In this study we examined the relevance of NFκB for colorectal cancer. In a series of 90 non-metastatic, lymphogenous-metastatic and haematogenous-metastatic colorectal carcinomas, we determined the expression pattern of NFκB in the context of the metastatic status of these tumors. Since we found significant up-regulation of cytosolic NFκB in tumor epithelium and its nuclear accumulation in dissociated tumor cells we examined a possible correlation between the expression levels of nuclear NFκB and the adhesion molecule E-cadherin in colorectal carcinoma specimens. We examined the reproducibility of the data obtained by in situ analysis in a 3D in vitro culture model using tumor spheroids, which more closely imitate the in vivo situation than conventional monolayer culture systems. Furthermore, in accordance with our previous data, suggesting that the bacterial lipopolysaccharide (LPS) causes up-regulation of adhesion molecules, which play an essential role in metastasis, we examined the influence of LPS stimulation on the expression of NFκB in various colorectal carcinoma cell lines (7).

Materials and methods

Cultivation of adherent tumor cells and LPS stimulation. The human colorectal cell lines SW837, HRT18, CX-1, CX-2, SW620, SW948, HT-29 and CaCo2 were grown in RPMI-1640 medium supplemented with Glutamax (Sigma), 10% heat deactivated FCS (Gibco), 1% penicillin (Gibco) and 1% streptomycin (Gibco) at 37°C in a humidified incubator containing 5% CO₂. LPS stimulation was performed by replacing the growth medium of subconfluent cultures with medium supplemented with LPS derived from E. coli (Sigma), 1 μg/ml. After 4 h the cells were harvested and used for preparation of extracts for expression analysis.

3D culture of HT-29 cells as spheroids. The HT-29 cells were harvested in the exponential growth phase (80% confluency). After washing once in medium the cells were seeded on agarose-coated (1%) 96-multiwell plates at a concentration of 1000 cells/200 μl. Within 5 days of incubation at 37°C the cells formed compact aggregates. For expression studies only cells in the plateau phase of growth were used. This state is reached after cultivation of the spheroids for two weeks. After two weeks the spheroids were harvested, formalin-fixed, embedded in paraffin and processed for immunohistochemistry.

Tissue samples. The colorectal tissue samples used in this study were obtained from 90 patients undergoing elective surgery for colorectal cancer at the University of Mainz during the years 1995-1999. The investigation of these tissues was in accordance with the rules of the responsible state ethics committee. The morphological classification of the carcinomas was conducted according to WHO specifications. The tumors were staged following the guidelines of the TNM Classification of Malignant Tumors. With respect to the T status all tumors investigated were T3 and moderately differentiated (G2) and were separated into three groups according to metastatic status. The first group included 30 cases without tumor metastasis to regional lymph nodes or distant organs (N0/M0). Among the remaining 60 metastatic cases 30 were characterized by lymphogenous (N+) and 30 by haematogenous metastases (M+). For all samples investigated follow-up data were obtained from hospital charts and by corresponding with the physicians in charge during a period of 5 years after surgery.

Antibodies. Primary antibodies: mouse monoclonal NF-κB (RelA) (F-6, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-E-cadherin (BioGenex) and rabbit polyclonal anti-ß-actin (Serva).

Western blot analysis. Protein extracts from harvested human cells and normal and tumor epithelia derived from patients undergoing surgical resection of colorectal carcinomas were prepared in TKM buffer (50 mM Tris, pH 7.5/150 mM KCl/5 mM MgCl₂) using a Dounce homogenizer. The total protein content was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH). For Western blotting aliquots from each sample containing 20 μg of total protein were separated on sodium dodecyl sulphate-polyacrylamide gels SDS-PAGE (10%) and then transferred to polyvinylfluoride (PVDF) membranes (Immobilen-P, Millipore Corp.) in accordance with standard procedures. Incubation with the primary antibodies to NFκB (RelA) and ß-actin was performed overnight by 4°C. Immunodetection was performed using the alkaline phosphatase (AP) conjugated anti-mouse and anti-rabbit IgG (Sigma). As AP substrate a mix containing 0.45% nitroblue tetrazolium (NBT) (Serva) and 0.35% bromo-4-chloro-3-indolyl phosphate toluidinium salt (X-Phosphat) (Serva) in AP buffer (100 mM NaCl/50 mM MgCl₂/100 mM Tris, pH 9.2) was used. Primary antibodies were used in a 1:100 dilution. Secondary antibodies were used at concentrations recommended by the suppliers.

Immunohistochemistry/cytochemistry. All immunohistochemical reactions were conducted using formalin-fixed and paraffin-embedded samples. After deparaffinization the samples were treated in a microwave oven in EDTA buffer for 15 min. Incubation with the primary antibodies to NFκB (RelA) and E-cadherin, and the secondary antibodies, horse anti-mouse biotinylated IgG (Vector Laboratories, Inc.) were carried out using the Vectastain Elite reagent (Vector Laboratories, Inc.). Anti-NFκB was used at a dilution of
The level of cytoplasmic NFκB is elevated in colon cancer epithelia. To elucidate the relevance of NFκB for colorectal cancer progression we determined its expression in non-metastatic (N0/M0, 3 cases) and metastatic (N+ or/and M+, 5 cases) colon carcinomas by Western blotting (Fig. 1) and by immunohistochemistry (Fig. 2). All specimens examined were derived exclusively from the surface of the tumor. Furthermore, all samples chosen for this analysis were comparable regarding the amount of the tumor cells and the adjacent tissue including the surrounding cells positive for NFκB. As shown in Fig. 1 all tumor cases are characterized by a significant increase of NFκB expression as compared to normal tissue. The up-regulation is independent of the metastatic status of the tumors. The non-neoplastic tissue of 6 of the cases investigated showed a weak to moderate NFκB expression whereas in 2 cases the staining for NFκB was negative.

To verify the data gained by Western blotting a large pool of colorectal carcinomas, 90 in all, was investigated by immunohistochemistry (Table I). The cases examined were grouped according to their metastatic status into three groups. The first group included 30 non-metastatic cases (N0/M0). The remaining 60 cases had metastases, 30 of these samples being characterized by lymphogenous (N+) and 30 by haematoogenous (M+) metastases. With respect to the T status all tumors investigated were defined as T3 and moderately differentiated (G2). This selection was performed with regard to a potential relationship between the expression level of NFκB and the metastatic status of the tumors. As shown in Table I all cases investigated were positive for cytoplasmic NFκB. Considering the expression intensity - weak, moderate, strong - the cases were classified into three groups (Table I, Fig. 2A-C). The data presented in Table I indicate that cancer progression in the colon correlates with increase of cytosolic NFκB expression. Whereas only 20% of the N0/M0 tumors showed high cytosolic NFκB levels, 43% (p=0.09) of the lymphogenous- and 47% (p=0.05) of the haematogenous-metastatic cases gave strong expression. In contrast, the non-neoplastic tissue revealed only a weak cytoplasmic immunostaining (Fig. 2D).

**Table I. Expression of NFκB (RelA) in non-metastatic (N0/M0), lymphogenous-metastatic (N+) and haemato- genous-metastatic (M+) colorectal carcinomas.**

<table>
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<tr>
<th>Colorectal carcinomas</th>
<th>n</th>
<th>Weak (%)</th>
<th>Moderate (%)</th>
<th>Strong (%)</th>
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<tr>
<td>N0/M0</td>
<td>30</td>
<td>7 (23)</td>
<td>17 (57)</td>
<td>6 (20)</td>
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<tr>
<td>N+</td>
<td>30</td>
<td>8 (27)</td>
<td>9 (30)</td>
<td>13 (43)</td>
</tr>
<tr>
<td>M+</td>
<td>30</td>
<td>7 (23)</td>
<td>9 (30)</td>
<td>14 (47)</td>
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‘Considering the expression levels, the cases examined were separated into three groups. Group 1 is characterized by a weak NFκB (RelA) expression (cf. Fig. 2A). Group 2 shows moderate NFκB (RelA) level (cf. Fig. 2B) whereas group 3 is characterized by a strong stain (cf. Fig. 2C). n, number of cases examined.'
staining nuclear NFκB localization in cells, which were still vital but dissociated from the surrounding cohesive tumor tissue (Fig. 3A2) undergoing cell death. This morphological finding implies a correlation between the activation of NFκB, loss of adhesion and the necrotic process. Since tissue integrity is principally mediated by the cell-cell adhesion molecule E-cadherin, we determined its expression in the aforementioned 65 cases. Interestingly, 43 (66%, p=0.0008) of these samples showed loss of E-cadherin (Table II and Fig. 3A3) (8). In a few samples loss of E-cadherin was observed within tumor complexes which were not associated with the central necrosis (Fig. 3B1-3). Here mainly an increase of cytosolic NFκB expression and nuclear staining of only a few tumor cells was found. Additionally, in 10 adenocarcinomas with local mucus production a positive nuclear NFκB expression was found in the E-cadherin-negative tumor cells present in the mucus (Fig. 3C1-3). The association of NFκB activation and loss of E-cadherin was independent of the metastatic status of the examined cases.

Nuclear expression of NFκB is associated with loss of E-cadherin in the HT-29 spheroid model. Adenocarcinomas of the colon are characterized by a typical morphological feature, namely central necrosis surrounded by neoplastic glandular nests. As described previously 3D spheroid cultures of tumor

<table>
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<tr>
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<th>NFκB+</th>
<th>NFκB-</th>
<th>Total</th>
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<tr>
<td>E-cadherin positive</td>
<td>6</td>
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<td>15</td>
</tr>
<tr>
<td>E-cadherin negative</td>
<td>43</td>
<td>7</td>
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</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>16</td>
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Figure 2. The level of cytosolic NFκB (RelA) is up-regulated in colorectal carcinomas. A, Specimen characterized by a weak stain. B, Example of a moderate stain. C, Specimen showing a strong immunoreactivity to NFκB (RelA). D, NFκB (RelA) expression in non-neoplastic colon mucosa. The sections were counterstained with Mayer’s hematoxylin. Magnification x400.

Figure 3. In colorectal carcinomas nuclear location of NFκB (RelA) expression correlates with loss of E-cadherin. A1, Morphology of a colon adenocarcinoma characterized by an area of central necrosis surrounded by neoplastic glandular nests stained with hematoxylin. A2, Nuclear NFκB (RelA) staining in still viable cells but dissociated from the surrounding cohesive tumor. A3, E-cadherin is expressed in the cohesive tumor but not in the dissociated cells positive for NFκB (RelA). B1, Compact tumor complex stained with hematoxylin. B2, Strong NFκB (RelA) staining is visible in the cytoplasm. A few cells show also nuclear staining. B3, Cells expressing NFκB (RelA) are negative for E-cadherin. C1, Adenocarcinoma with local mucus production stained with hematoxylin. C2, Cells floating in mucus are positive for nuclear NFκB (RelA). C3, Cells expressing NFκB (RelA) are negative for E-cadherin. Magnification x160 (A1) and x400 (A2-C3).
cells simulate precisely the in situ morphology of colorectal carcinomas including loss of cell-to-cell interactions (9). Thus, we used this model to confirm the in situ correlation between increase of NFκB and loss of E-cadherin expression. As shown in Fig. 4A in the inner part of HT-29 spheroids a necrotic centre is formed during the plateau growth phase simulating the in situ situation. B1 and B2, NFκB (RelA) is expressed in the cytosol of the cells forming the compact region of the spheroid (B1) and in the nucleus of a few dissociated cells destined to die (B2). C1 and C2, E-cadherin is expressed in the membrane of the tightly organized cells (C1) and lost in the dissociated cells (C2). Magnification x160 (A1) and x400 (A2-C2).

Figure 4. Nuclear expression of NFκB (RelA) is associated with loss of E-cadherin in the HT-29 multicellular spheroid model. A1 and A2, HT-20 spheroid (A1) and a section of it (A2) showing the compact cell area and the dissociated cells stained with hematoxylin. Around the central necrosis a non-proliferative area with dissociated but still viable cells is formed in the plateau growth phase simulating the in situ situation. B1 and B2, NFκB (RelA) is expressed in the cytosol of the cells forming the compact region of the spheroid (B1) and in the nucleus of a few dissociated cells destined to die (B2). C1 and C2, E-cadherin is expressed in the membrane of the tightly organized cells (C1) and lost in the dissociated cells (C2). Magnification x160 (A1) and x400 (A2-C2).

Figure 5. Detection of NFκB (RelA) in non-stimulated and LPS-stimulated human colorectal carcinoma cell lines by Western blotting. Aliquots of cell homogenates containing 20 μg of total protein were loaded onto the SDS-PAGE gel (10%) and after separation transferred to PVDF membrane. Staining with anti-β-actin was performed as a control for loading.
membranous staining of this adhesion molecule is visible (Fig. 4C).

**LPS induces NFκB-expression in colorectal carcinoma cells.** Since exposure of certain cell types to LPS leads to activation of NFκB, we asked whether this is true also for cell lines derived from colorectal carcinomas (2). We examined by Western blot the expression of NFκB in non-stimulated and LPS-stimulated SW837, HRT18, CX-1, CX-2, SW620, SW948, HT-29 and CaCo2 cells. As shown in Fig. 5 all eight cell lines are positive for NFκB. Its expression level differs from very weak in the well differentiated CaCo2 cells to moderate or high in the less differentiated cell lines. Interestingly, LPS treatment of the CaCo2 cells, characterized by lower constitutive NFκB levels, caused a marked increase of its expression (Fig. 5). In the cell lines characterized by relatively high amounts of constitutive NFκB a significant response to LPS was also observed. To elucidate whether LPS stimulation influences not only the expression level but also the subcellular localization of NFκB we examined its distribution in CaCo2 cells by immunocytochemistry. Considering the kinetics of the translocation process we monitored the expression pattern at five different time-points (0, 30 min, 1, 2 and 4 h after LPS exposure). As shown in Fig. 6 in both the non-stimulated (A) and LPS-stimulated CaCo2 cells (B and C) only a cytoplasmatic staining is apparent. A significant increase of NFκB level was visible already one hour after stimulation.

**Discussion**

The pivotal role of the transcription factor NFκB in cell proliferation and survival has been documented by numerous studies. Since both processes are strongly associated with neoplastic transformation and progression of malignancies, the finding that NFκB expression is altered in diverse neoplastic cells and tissues derived from both hematopoietic and solid tumors was not surprising. Currently it is known that both NFκB and its regulators are associated with tumor-related processes, such as neoplastic transformation, proliferation, apoptosis, angiogenesis and metastasis. However, the mechanisms leading to its aberrant activation/silencing and cellular trafficking are not understood in the special context of defined cancers. Furthermore, the triggers of these processes have not yet been identified.

In this study it is demonstrated that colorectal adenocarcinomas are generally characterized by elevated levels of the inactive cytosolic NFκB. Nevertheless, this phenomenon was found to be strongly linked to the metastatic status of the tumors investigated, as both the lymphogenous- and haematogenous-metastatic tumors were affected. Thus, we suggest that up-regulation of NFκB is associated with the aggressiveness of the tumors. Nuclear localization of the transcription factor was detected exclusively in cells dissociated from the central necrosis of the adenocarcinomas. Our results confirm the predominant cytosolic and exclusively focal nuclear localization of NFκB found in colorectal, pancreatic, gastric and prostate carcinomas by other investigators (10-15). In agreement with our suggestion of a correlation between the up-regulation of NFκB and the aggressiveness of the tumors, Evertsson and Sun found NFκB activation in mucinous colorectal adenocarcinomas with a poor prognosis and Kojima et al in the more progressed T3+T4 cases (10,16). Additional support is provided by correlation of NFκB and VEGF expression, suggesting a link between NFκB and tumor angiogenesis (12). NFκB inhibitory action in apoptosis has also been found in colorectal carcinomas (11,17). With regard to the nuclear staining of the dissociated cells in the vicinity of the central necrosis the question arises whether the translocation of NFκB into the nucleus results directly from loss of homeostasis between the active and the non-active form of the transcription factor. Furthermore, whether this loss of balance is due to the aberrant up-regulation of NFκB expression and which molecular/genetic events are responsible for it, remain unclear.

Previously, we identified LPS, the endotoxin of intestinal Gram-negative bacteria, as a pivotal stimulus increasing the metastatic potential of colorectal cancer (7,18). Therefore, in the context of the present studies the expression of NFκB was examined in colorectal tumor cell lines SW837, HRT18, CX-1, CX-2, SW620, SW948, HT-29 and CaCo2 after LPS treatment. Since in all cases an increase in NFκB level has been observed, LPS may act *in situ* as a trigger of tumorigenic processes associated with NFκB up-regulation. The association of LPS-mediated NFκB activation with metastasis has already been described by Luo et al (19). In an experimental murine tumor model LPS stimulation of a colon adenocarcinoma cell line caused generation of lung metastases. The metastatic growth response has been found to depend on NFκB activation in the tumor cells. The inhibition of NFκB activity converted the growth response into a death response. Andrews et al found in the human metastatic colon cancer cell line LS174T that exposure to LPS increases the adhesion of the tumor cells to the endothelium through an NFκB-dependent pathway (20).

As mentioned above, nuclear NFκB localization was observed only in cells dissociated from the cancer nests. Thus, we assumed an association between the active form of NFκB and loss of tissue integrity caused by loss of cell to cell contacts. Indeed, it has been demonstrated here that the nuclear localization of NFκB is associated with down-regulation of the adhesion molecule E-cadherin. These *in situ* results were confirmed *in vitro* using a 3D-spheroid model of HT-29 cells. In keeping with our findings, a link between function(s) of NFκB and E-cadherin has been suggested by others (21,22). In a mammary tumors model a correlation of activation of NFκB and loss of E-cadherin was found in cells of the more invasive phenotype (21). Activation of NFκB caused by loss of E-cadherin was found in melanoma cell lines (22). However, our studies show that the correlation between loss of E-cadherin and up-regulation of NFκB concerns a defined area of the tumor, namely, dissociated cells around the central necrosis of the cancer nests, suggesting that the correlation between NFκB activation and loss of E-cadherin may be directly associated with induction of tumor necrosis in colorectal cancer. Considering the fact that cancer cell necrosis plays a crucial role in tumor progression this observation is of great importance. It is known that necrosis of tumor cells that are located in the hypoxic core of the tumor leads to an activation of tumor-
associated macrophages (TAM). These in turn release growth, survival and angiogenic factors that support tumor progression and increase tumor angiogenesis and invasion (2,23). Furthermore, the induced hypoxia potentiates these processes. However, according to the ‘cross-priming’ hypothesis the release of necrotic cells can reduce tolerance to tumor antigens, thereby potentiating immunosurveillance and potentially repressing tumor growth. Thus, tumor necrosis seems to have a dual and, interestingly, opposing role in the complexity of tumor events.

In conclusion, our data show that the transcription factor NFκB is of great importance for colorectal cancer. Both non-metastatic and metastatic tumors show up-regulation of cytosolic NFκB as compared to normal epithelium. Furthermore, since treatment of in vitro cultivated cells with LPS results in increase of NFκB levels, this endotoxin must be taken into consideration as a potential trigger of this process in situ. The increase of the cytoplasmic NFκB level correlates with the metastatic potential of the tumors, suggesting that the up-regulation of NFκB may be relevant to metastasis. In addition, nuclear NFκB expression strongly correlates with loss of E-cadherin expression. This correlation mainly concerns tumor cells dissociated from the necrotic core of cancer nests, thus, linking it to loss of tissue integrity and tumor necrosis, both of which are essential features in tumor progression.

References