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14. ABSTRACT

Bisphosphonates (BPs, i.e. clodronate and zoledronate) are frequently used in the treatment of breast cancer, once bone metastases have occurred. These drugs inhibit cancer-induced bone complications, because they inhibit the bone resorbing osteoclasts. Less is known about the direct effects of BPs on breast cancer cells. The aim of this work was to study the possibility that some of the BP-induced effects in breast cancer cells might be mediated via the Toll-like receptor pathway. We discovered that human breast cancer cell lines exhibit variable expression of Toll-like receptor 9 (TLR9). Expression of TLR9 was associated with increased invasiveness upon stimulation of the cells with TLR9 agonists. Since similar effects were also seen in other types of cancer cells that express TLR9, this may represent a novel mechanism through which bacterial infections promote cancer progression.

15. SUBJECT TERMS
Toll-like receptor, bisphosphonates, breast cancer

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Table of Contents

Introduction

Bisphosphonates (BPs), such as clodronate and zoledronate, have been traditionally added to the treatment of breast cancer patients once bone metastases have occurred.[1] Bisphosphonates inhibit cancer-induced bone complications, because they inhibit the bone resorbing osteoclasts, whose activity the cancer cells residing in bone typically activate.[2] Recent animal studies have suggested, however, that BPs given in a preventive manner may reduce cancer spread to bones.[3] Results from recent clinical studies, where clodronate was added to conventional chemo- and/or radiation therapy of breast cancer patients upon diagnosis, have been conflicting.[4-6] In one study, patients with estrogen receptor negative (ER-) breast cancer exhibited increased local recurrence of the tumor, increased visceral metastases and decreased survival.[5] The cell biological basis for these findings is not known. They do suggest, however, that further studies are needed to clarify all the unknown effects of BPs on breast cancer cells, before these drugs can be used as a standard adjuvant therapy. We have shown previously that BPs activate the p38 MAP kinase pathway in breast cancer cells.[7] This activation confers the cells resistance against the BP-induced growth inhibition because blocking p38 results in the augmentation of the anti-growth effects of BPs. The upstream events of this activation are not known. It was also shown previously that BPs inhibit the invasion of prostate and breast cancer cells. [8]

The aim of this work is to study the possibility that some of the BP-induced effects in breast cancer cells might be mediated via the Toll-like receptor pathway. This is because there are similarities between the responses to BPs and Toll-like receptor ligands in cells.[9, 10]

BPs may be becoming a gold standard adjuvant treatment for breast cancer patients.[11] Before such a change in the clinical practice can be made, it is important to identify the mechanisms that these drugs use in cells. Based on the preclinical findings, it is also important to find the patient populations who might actually suffer from the use of BPs. Our research may help in defining such patient populations whom should and should not be treated with BPs. Furthermore, our results may lead the way to the development of better BP-molecules, which would lack the cancer cell growth promoting activity.

Body

Task 1. **Establish the concept of a "bisphosphonate-receptor" in human breast cancer cells**

a. Culture human breast cancer cell lines with high doses of nitrogen-containing BPs (n-BPs, 10^{-4} M zoledronate or pamidronate), to induce decrease in cell viability, as detected with MTS-assays. Show that this decrease in cell viability can be blocked with 10X-excess of another class of BPs (pyrophosphate-containing BPs, p-BPs *i.e.* clodronate and etidronate), which by themselves do not kill breast cancer cells.

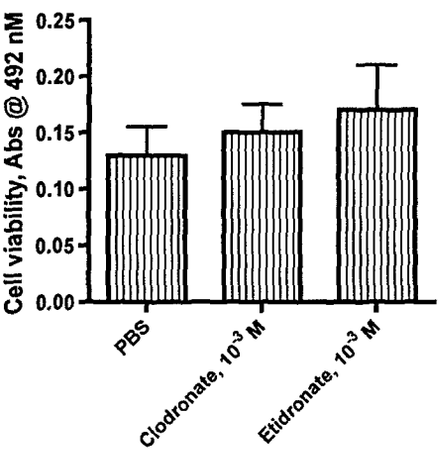
b. Culture the above mentioned human BC cell lines with n-BPs (10^{-4} M zoledronate or alendronate), to induce an accumulation of unprenylated GTP-binding proteins, such as Rap1A, which can be detected with specific antibodies in Western blots. Use this method as a surrogate marker for n-BP inhibition of the mevalonate pathway in BC cells. Then show that this effect of n-BPs can be blocked with 10X-excess of p-BPs, which by themselves do not inhibit the mevalonate pathway in cells and do not thus induce accumulation of unprenylated Rap1A. Together these experiments should establish that 1) Excess p-BPs can block the effects of n-BPs in BC cells and vice versa, suggesting that these two classes of BPs compete for the same uptake mechanism is cells.

Results:

We were indeed able to block the effects of one type of a bisphosphonate (n-BP, pamidronate, alendronate, risedronate), with another type of a bisphosphonate (p-BP, clodronate, etidronate), suggesting that there is a receptor or a cellular transport mechanisms for which the two classes of BPs compete. These findings are demonstrated in Figure 1, where we show that clodronate and etidronate per se do not affect cell viability, but when given with the n-BPs, they prevent the decrease in cell viability that is induced by n-BPs. (Figs 1a & b). Clodronate also inhibited the accumulation of unprenylated Rap1A, which was induced with alendronate (Fig. 1 c.). These findings suggest that p-BPs inhibits the entry of n-BPs into the mevalonate pathway.

FIGURE 1.

a.


Cell viability, Abs @ 492 nM
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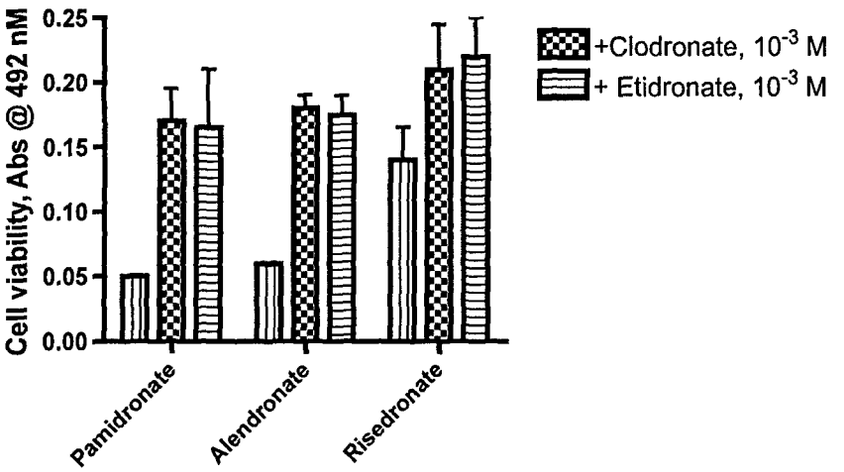
Cell viability, Abs @ 492 nM
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Alendronate
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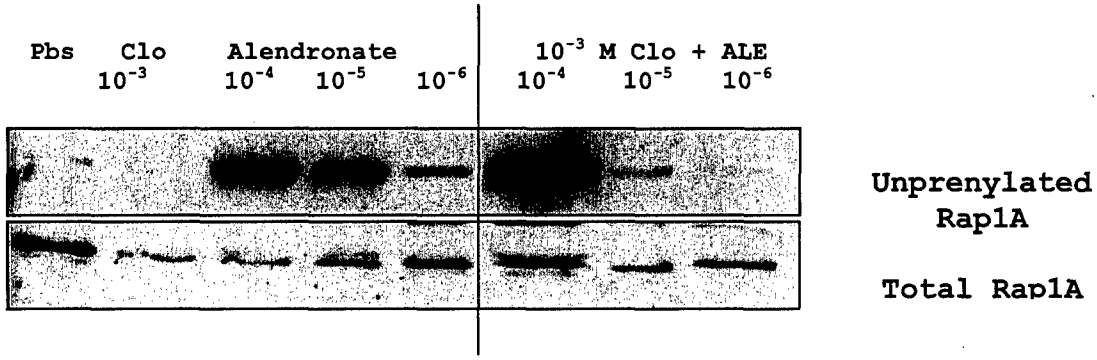
+ Etidronate, 10-3 M

c.



Task 2. **Study the expression of TLR1-10, MyD88, IRAK and TRAF-6 in human BC cell lines**

Results:

We discovered that breast cancer cells exhibit different levels of expression of various TLRs and that especially TLR9 is highly expressed in some, but not all breast cancer cells. Interestingly, TLR9 mediates invasion in breast cancer cells. (Please see the attached manuscript).

Task 3. **Prepare TLR downstream signaling molecule "knockout" BC cells.**

Results: We are in the process of doing these experiments.

Task 4. **Study BP-responses in TLR "knockout" and control BC cells**

Results: We are in the process of doing these experiments. Our results do suggest, however, that TLR9 may mediate the BP-induced and p38-mediated effect, which keeps the cells in the cell cycle. This is suggested by the finding that the TLR9 siRNA knockout MDA-MB-231 cells are more sensitive to the anti-proliferative effects of zoledronate, which is a powerful new bisphosphonate (Fig. 2). Interestingly, however, accumulation of unprenylated Rap1A was not affected in the TLR9 siRNA knockout cells (data not shown).

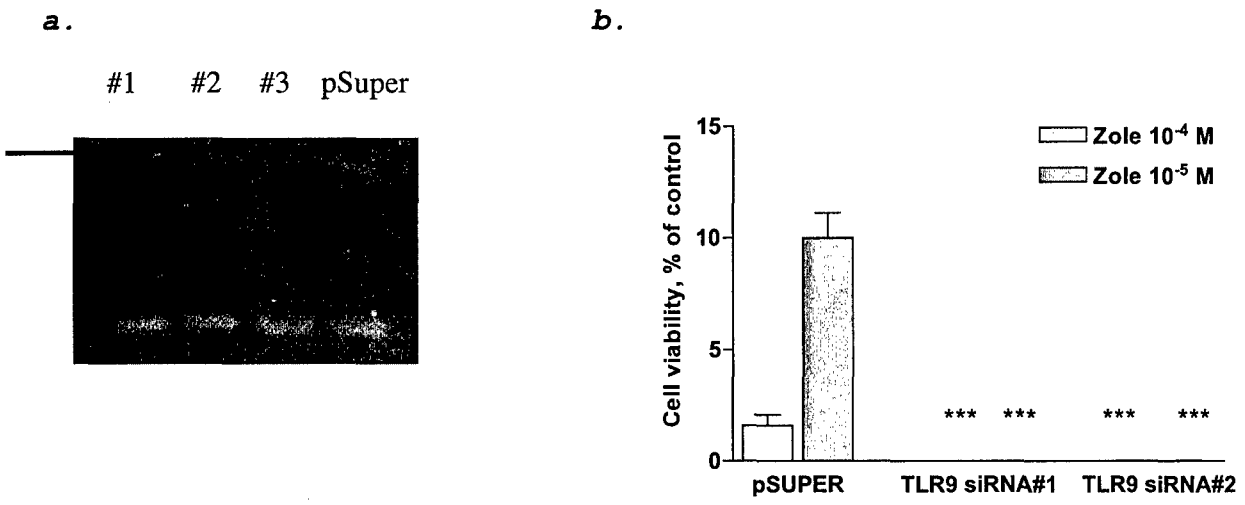


Figure 2. TLR9 siRNA knockout breast cancer cells exhibit increased sensitivity to BP-induced cell death.
A) Human MDA-MB-231 breast cancer cells were transfected with pSUPER TLR9-siRNA constructs (#1, #2, #3) or with the empty vector pSUPER. Lack of TLR9 expression from the TLR9-siRNA pools #1, #2 and #3(representing separate siRNA constructs) was confirmed with RT-PCR and only the pSuper empty vector pool had TLR9 expression (arrow). B) The cells were then cultured with zoledronate (10^{-4} or 10^{-5} M) or PBS as a vehicle control for 5 days and cell viability was measured with a standard MTT-assay. The pSUPER empty vector control cells exhibited a dose response to zoledronate-induced cell death with the least viability detected with the highest zoledronate dose. The TLR9-siRNA clones instead did not survive at any zoledronate dose tested. Data is expressed as % of PBS-control. Mean ± sd, n= 6. *** p <0.001 vs. the corresponding pSUPER clone.

Task 5. Study changes in TLR-pathway proteins in response to BP-treatment

Results: Our preliminary data suggests that BPs up-regulates IL-8, similar to CpG-oligonucleotides, which are ligands for TLR9 (data not shown). We are in the process of analyzing subcellular expression of the various TLR-pathway proteins.

Key research accomplishments

-We have discovered that breast cancer cells express variable amounts of TLR9
-Expression of TLR9 is associated with the ability of these cells to respond to TLR9-treatment
-Treatment with TLR9-agonists increases the invasion of cancer cells
-Our findings may represent a novel pathophysiological mechanism through which infections promote cancer progression
-We have also data to suggest that some of the bisphosphonate-mediated effects in breast cancer cells, such as the drug-induced cell cycling- is mediated via TLR9

Reportable outcomes

Submitted manuscripts:

Toll Like Receptor-9 Agonists Promote Invasion Of Human Breast Cancer Cells By Increasing Matrix Metalloproteinase-13 Activity; Melinda A. Merrell, Niko Lehtonen, Timo Sorsa, Bradley Gehrs, Eben Rosenthal, Dongquan Chen, Brit Shackley, Kevin W. Harris, Katri S. Selander

Presentations:

Toll Like Receptor-9 Agonists Promote Invasion Of Human Breast Cancer Cells By Increasing Matrix Metalloproteinase-13 Activity; Melinda A. Merrell, Niko Lehtonen, Timo Sorsa, Bradley Gehrs, Eben Rosenthal, Dongquan Chen, Brit Shackley, Kevin W. Harris, Katri S. Selander

ERA of Hope, Philadelphia, June 8-11, 2005

Employment:

Merrell MA (research assistant)
Selander KS (principal investigator)

CONCLUSIONS:

Importance

We have discovered a novel mechanism through which bacterial infections may promote cancer progression. The manuscript in which these findings are reported has been submitted to Cancer Cell.
Since bisphosphonates have been shown to inhibit cell invasion, it is possible that they act on the TLR9-mediated invasive pathway. These experiments are ongoing in our laboratory.

"So What"

There is evidence to suggest that bacterial infections and the ensuing inflammation promote cancer growth. Usually, this is though to be mediated solely via the inflammation mediating cytokines. We discovered that not only the inflammatory cells, but also cancer cells may express Toll-like receptors. Furthermore, stimulation of the Toll-like receptor 9 results in increased invasion of the cancer cells. These findings offer new explanations for pathophysiological alternatives in infection-promoted cancer. Furthermore, this is the first time that TLR9 has been linked to cancer invasiveness and therefore, these results show that TLR9 can be a new molecular target for the development of cancer drugs.

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Appendix:

Merrell MA & al., submitted manuscipt

Toll Like Receptor-9 Agonists Promote Invasion Of Human Breast Cancer Cells By Increasing Matrix Metalloproteinase-13 Activity

Melinda A. Merrell [1], Niko Lehtonen [2], Timo Sorsa [2], Bradley Gehrs[1], Eben Rosenthal [3], Dongquan Chen[4], Brit Shackley[1], Kevin W. Harris [1,5], Katri S. Selander [1*]

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Summary

Toll-like receptor 9 (TLR9) recognizes microbial DNA in cells. We show here that TLR9 protein is expressed in human breast cancer cells to a variable degree. TLR9 agonistic CpG-oligonucleotides (CpG-ODNs) dramatically stimulated the *in vitro* invasion of TLR9 expressing (TLR9+), but not that of TLR9 – breast cancer cells. Similar effects on invasion were seen in TLR9+ astrocytoma and glioblastoma cells after CpG-ODN-treatment. The CpG-ODN-treatment induced formation of a ~55 kDA gelatinolytic band in zymograms and increased invasion were abolished by a matrix metalloproteinase (MMP) inhibitor. Significantly increased MMP-13 levels were detected from supernatants of CpG-ODN-treated breast cancer cells. Neutralizing antibodies against MMP-13, but not control IgGs, inhibited the CpG-induced invasion *in vitro*.

Significance

Chronic infections are considered to be among the most important environmental factors contributing to tumor progression, but the mechanisms have remained unclear. We have identified a new molecular mechanism through which bacterial infections may promote cancer invasion. Through this mechanism, stimulation of the intracellular TLR9 receptor of the innate immune system with bacterial DNA can directly promote cancer invasion via activation of MMP-13. These findings provide novel clues to why chronic infections may promote cancer and metastasis in general. They also offer a new molecular target for cancer therapy, since TLR9 has not been associated with invasiveness previously. Our results also warrant cautiousness in the use of TLR9 agonists in cancer immunotherapy, since functional TLR9 may be expressed by cancer.

Introduction

Toll-like receptors (TLRs) are evolutionarily well conserved trans-membrane proteins which are present in almost all multi-cellular organisms and recognize patterns specific of microbial components.[1, 2] In mammals the TLR family is currently known to consist of 11 members, which exhibit specificity for pathogen-derived ligands. For example, TLR4 recognizes the bacterial lipopolysaccharide (LPS), whereas members of the TLR9 subfamily (TLRs 7,8,9) sense the microbial RNA and DNA.[1, 2] Oligonucleotides with unmethylated CpG dinucleotides mimic the immunostimulatory activity of bacterial DNA in vertebrates and are also recognized by TLR9.[3-6]

TLRs 1,2 and 4 are expressed on the cell surface whereas TLR3 and members of the TLR9 subfamily are intracellular.[7-10] More specifically, TLR9 is localized to endoplasmic reticulum, from where it is translocated to the endosomal/lysosomal compartment for ligand recognition.[10] Upon ligand binding, the various TLRs and their associated adapters, such as MyD88 and TRIF, recruit intracellular signaling mediators which activate transcription factors, such as NF-κB. The outcome of TLR activation is an immune reaction, characterized by increased production of various pro-inflammatory cytokines and interleukins.[2]

In humans, TLR9 is most abundantly expressed in plasmocytoid dendritic cells and in B cells whereas in mice, also myeloid dendritic cells as well as macrophages and B cells express TLR9. Interestingly, several epithelial cell types and astrocytes have also recently been reported to express various TLRs, implying that also other than immune cells may be important sentinels of the innate immune system.[9, 11-14] High expression of TLR9 was recently detected in clinical samples of lung cancer and in lung cancer cell lines. In these cells stimulation of TLR9 with its agonists was shown to result in the production cytokines.[15] Responsiveness of breast cancer cells to TLR ligands and the presence of TLRs in breast milk suggest that they are also expressed in breast epithelial cells.[16, 17]

The aim of this study was to further characterize TLR expression and function in human breast cancer cells. Since the original observation about the role of Toll in Drosophila was that stimulation of the TLR pathway increases cell migration, we hypothesized that TLRs may mediate similar effects also in breast cancer cells. [18]

Results and Discussion

Breast cancer cells express TLR9. MDA-MB-231 cells express relatively high levels of mRNAs for TLR4 and TLR9, whereas only very little or no mRNAs for the other TLRs 1- 10, as detected in DNA-arrays. Flow cytometry of the permeabilized MDA-MB-231 cells suggested intracellular expression of TLR9, as also shown previously in other cells. [2, 19] Anti-TLR9 antibody detected a high level of expression of a band ~120 kDa in MDA-MB-231 cells and an intermediate level in T47-D cells, but no specific signal was seen in MCF-7 cells, in Western blots (Figure 1.).

TLR9 agonists induce invasion in TLR9 expressing cancer cells. To study the effects of TLR9 stimulation on breast cancer behavior, we performed cell viability and invasion assays using the well-characterized TLR9 agonists, the CpG-motif containing unmethylated oligonucleotides (CpG-ODN).[2] No effects on cell viability were detected when the cells were cultured for 24 h with 10 μM CpG-ODN, as studied with MTS-assays. The CpG-ODNs induced, however, a significant, dose-dependent increase in the number of MDA-MB-231 cells that invaded through Matrigel. Similar effects were seen MDA-MB-231 cells were cultured for 7 days in the presence of 10 μM CpG-ODNs in 3-dimensional collagen culture assays. CpG-ODNs stimulated invasion also in the unrelated, strongly TLR9-expressing U373 astocytoma and D54MG glioblastoma cells and in T47-D breast cancer cells. Interestingly, CpG-ODNs did not stimulate invasion in the TLR9 negative MCF-7 breast cancer cells. To further investigate whether the finding was specific to TLR9 agonists we also performed invasion studies using the TLR7- and TLR4-agonists, loxoribine and LPS, respectively. LPS, but not loxoribine induced invasion of MDA-MB-231 cells in Matrigels (data not shown). Not all CpG-ODN induced responses are TLR9 mediated. For example, it was recently shown that CpG-motif containing DNA activates the Akt pathway and this is dependent on the DNA-dependent protein kinase, but not TLR9.[20] Furthermore, plasmid DNA containing CpG-motifs elicited similar immune responses in TLR9 -/- and in TLR9 +/+ mice.[21] The fact that we saw increased invasion only in TLR9 expressing cancer cells, but not in TLR9 negative cells, however, strongly argues that the effect on invasion is mediated

via TLR9. In Drosophila, Toll was originally identified as a transmembrane receptor required for the establisment of dorso-ventral polarity in the developing embryo. [22] It was also found to be important in Drosophila immunity, as flies lacking this protein were highly susceptible to infection with Aspergillus fumigatus.[23] Therefore, it seems that both these functions, the ability to recognize and fight against infectious micro-organisms and the ability to mediate invasion, appear evolutionarily well conserved between Drosophila and mammalian epithelial cells.

TLR9 agonists induce matrix metalloproteinase activity. To investigate the mechanism behind the TLR9-agonist-induced invasion, we performed gelatin-zymogram assays. Supernatants from MDA-MB-231 cells that were treated with 5 or 10 μM TLR9 agonists for 24 h induced the formation of a gelatinolytic band of ~55 kDa, which was not inhibited with the serine protease inhibitor aprotinin, but which did disappear when the gels were incubated with the broad spectrum matrix metalloproteinase inhibitor GM6001. Consistent with these findings, the CpG-induced invasion of MDA-MB-231 cells was also inhibited by the MMP inhibitor, but not by aprotinin in Matrigel-assays (Figure 3.).

TLR9 agonist-induced invasion can be blocked with neutralizing anti-MMP-13 antibodies. Based on the size of the CpG-ODN-induced gelatinolytic band, we hypothesized that CpG-ODN-treatment induces the activation of MMP-13. [24] Analysis of the supernatants of MDA-MB-231 cells treated either with vehicle or with 10 μM CpG-ODNs with an ELISA indeed revealed significantly increased MMP-13 activity in the TLR9-agonist treated supernatants, as compared with those of vehicle treated cells. Neutralizing antibodies to MMP-13 also blocked TLR9-agonist induced invasion, whereas control antibodies did not. Taken together, these findings suggest that TLR9 agonists stimulate breast cancer invasion via activating MMP-13. CpG-ODN treatment did not, however, result in increased expression of MMP-13. The TLR9 agonists did not affect the expression of Pai-1, Pai-2 or TIMP-1 either (data not shown). Therefore, CpG-ODNs activate MMP-13 in a yet unknown mechanism (Figure 4.). Interestingly, treatment of mouse astrocytes with CpG-ODNs resulted in increased expression of MMP-9. [4] Therefore, it seems that TLR9 agonist-induced MMP-profile is eventually cell specific.

TLR9 mediated invasion may represent a novel mechanisms through which infections promote cancer progression. Epigenetic and environmental factors, such as infections and ensuing inflammation are important regulators of tumor progression.[25] The innate immune system can promote tumor development and progression through inflammation-dependent mechanisms.[25, 26] For example, chemokines and cytokines derived from the immune and inflammatory cells can affect dramatically the host microenvironment and cancer cell behavior, resulting in increased growth and metastasis. Mediators of the innate immunity may also modulate the invasive capacity of cancer cells directly. Endotoxin/lipopolysaccharide (LPS), a cell wall constituent of Gram-negative bacteria has been shown to promote metastasis in a mouse model of colon cancer. [27, 28] In addition to the cytokine-mediated indirect effects on tumor progression, LPS-induced effects on metastasis may also be mediated via direct, increased invasiveness of the cancer cells, which frequently express TLRs. [28] We describe here a novel, direct mechanism through which TLR9, a receptor for bacterial DNA, may enhance cancer invasiveness. Our finding has several important implications. First, these findings may give novel clues to why certain infections, such as Mycoplasma might promote cancer progression.[29] Mycoplasma infections have been detected in various cancers, including breast cancer.[30, 31] After having been taken up by the cells, Mycoplasma bacteria have been detected in the same subcellular localization, the lysosome, where also activated TLR9 is localized.[19, 32] The identical subcellular location of TLR9 and Mycoplasma at least in theory facilitates the possibility that binding of Mycoplasma DNA to the cellular TLR9 could result in increased invasion. Second, CpG-ODNs are being tested in pre-clinical models as adjuvants for cancer immunotherapy. [33] Even though the concentrations of CpGs that have been used to treat mice in the various murine tumor models, where these compounds were shown to have anti-cancer efficacy possibly through dendritic cell activation, are much lower than the doses used here, our findings warrant caution in the use of immunomodulatory TLR9 agonists in the treatment of cancer and suggest for screening of TLR9 expression of the tumors. [34, 35] Finally, a recent study revealed a connection between an increased risk for the development of prostate cancer and a sequence variant of TLR4.[36] It is thus possible that similar

alterations in the TLR9 gene might also explain the individual's susceptibility to cancer progression .

Experimental procedures

Chemicals. Phosphorothioate modified CpG-ODNs (CpG oligonucleotide type C, human TLR9 ligand, ODN M362) and loxoribine were purchased from InVivoGen (San Diego, CA) and dissolved into endotoxin-free sterile d-H_2O per manufacturer's suggestion. TLR-pathway specific DNA-arrays were from SuperArray (Frederick, MD). Matrigels were from BD Biosciences (Bedford, MA), LPS was from Sigma (St. Louis, MO), aprotinin and MMP-inhibitor GM6001 were from EMD Biosciences (La Jolla, CA).

Cell culture. Human MDA-MB-231 breast cancer, U373 astrocytoma and D54MG glioblastoma cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin/streptomycin and non-essential amino acids (all from Gibco BRL, Life Technologies). T47-D cells were cultured in RPMI, and MCF-7 cells were cultured in MEM, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine and with 10 µg/ml insulin (Sigma, St.Louis, MO). All cell cultures were done in incubators in a 37°C atmosphere of 5% CO_2/95% air.

***Cell viability* assays.** MDA-MB-231, T47-D or MCF-7 cells were plated at the density of 1000 cells/well in 96-well plates in normal culture medium, and cultured for 24 h with 10 µM CpG-ODN or vehicle. Cell viability was assessed after MTS was added for the final 2 hours of the experimental cultures as recommended by the manufacturer (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI).

Western blotting. The cells were cultured on 6-well plates in their normal culture medium until near confluency, after which they were rinsed with sterile PBS and cultured for further 24 h in serum-free culture medium. The culture medium was then discarded and the cells were

harvested in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, Cell Signaling, Beverly, MA) and clarified by centrifugation. After boiling the supernatants in reducing SDS sample buffer for 5 minutes, equal amounts of protein (~50 μg) were loaded per lane and the samples were electrophoresed on 10% polyacrylamide SDS gel and transferred to a nitrocellulose membrane. TLR9 was detected with anti-TLR9 antibody (IMG-431, Imgenex, San Diego, CA). The same blots were stripped and re-blotted, using anti-actin antibody (Sigma), to show equal loading. The protein bands were visualized by chemiluminescence using SuperSignal West Pico ECL kit (Pierce, Rockford, IL).

TLR mRNA expression profiling. The mRNA expression levels of the various TLRs in MDA-MB-231 cells was investigated using the SuperArray human TLR-pathway specific gene expression profiling system (SuperArray Bioscience Corp., Frederick, MD). Briefly, total cellular RNA, was isolated using the RNAZol reagent (Tel-Test Inc., Friendswood, TX) from the cells grown in normal culture medium and converted to a labeled cDNA probe. The denatured cDNA was hybridized overnight at 60°C to nylon membrane that contained the target cDNAs. Chemiluminescence was used to detect the hybridization signal on a X-ray film (Eastman Kodak Company, Rochester, NY). Per manufacturer's instructions, the X-ray film was scanned with a high resolution scanner (~300 dpi) into a JEPG-format image, converted into a TIFF-format (8-bit inverted grayscale) image by using a software Photoshop (Adobe Systems Inc. San Jose, CA). The images were then uploaded into a software ScanAlyze (Eisen Lab, UC at Berkeley) to produce a raw intensity data sheet. The raw data from both the control and the treated groups were combined and uploaded into a software GEarrayAnalyzer (SuperArray Inc., Bethesda, MD), where differences and ratios between the treated and the control groups were analyzed. Background was subtracted from signals and a house-keeping gene such as actin was used to calculate the ratio.

Flow-cytometry. MDA-MB-231 cells were cultured on Petri-dish (∅ big ones cm) until ~ 70 % confluent. The cells were then detached using CellStripper (Fisher Scientific), and prepared for analysis using the BD Cytofix/Cytoperm Kit (BD Biosciences, San Diego, CA), according to the manufacturer's recommendations. Briefly, ~ 1 x10 ^{6} cells were suspended into 0.5 ml of

WHAT. PE-conjugated anti-human TLR9 antibody (eBioscience, San Diego, CA) or PE-conjugated, isotype controlled IgG was added to the cells (7 μl per tube). After incubation for 30 min at 4°C, the cells were rinsed twice with PBS, and analysed with FACS.

***In vitro* invasion assays.** For the Matrigel-invasion assay the cells were plated at the density of 5 x 10^4 (MDA-MB-231, U373, D54MG), 15 x 10^4 (T47-D) or 30 x 10^4 (MCF-7) cells per upper well in 750 μl of normal culture medium. Indicated concentrations of the CpG-ODNs or vehicle were added to both the upper and lower wells. When indicated, aprotinin (2 μM), GM6001 (2 μM), control IgG or neutralizing anti-MMP-13 antibody was added to both upper and lower wells. The cells were allowed to invade for 18 h, after which the inserts were removed and stained with Hema 3 stain set (Fisher Diagnostics, Middletown, PA), according to the manufacturer's recommendation. The number of invaded cells were counted from preselected 5 microscopic fields using a 40X objective. To assess invasion in a three-dimensional type I collagen gel, acid solubilized type I collagen (0.9 ml) was added to the Costar Transwell dishes (Corning, Inc., Corning, NY) and gelled over 45 min at 37°C. The collagen was prepared using rat-tail type I collagen dissolved in 0.2 % acetic acid at 3.2 mg/ml and gelled by neutralizing the acid with 0.3N NaOH containing phenol red as a pH indicator. A final concentration of 3.0 mg/ml was obtained. Media, containing vehicle or 10 μM CpG-ODN was then added to the upper and lower chamber prior to the addition of 5 x 10^5 cells to the surface of the collagen gel in the presence of serum-containing medium. Media were changed every three days over the 7-day incubation period. Gels were then removed from the Transwell dish, fixed in 2.7% formaldehyde for 24 h and embedded in paraffin. Sections (6-μm) were cut and stained with hematoxylin and eosin. Tumor cell invasion was assessed by light microscopy in a minimum of four randomly selected sections for each experimental sample. The number of invading cells per high power field (400X) were counted and averaged.

Zymograms. The zymograms were performed as previously described.[37] Briefly, MDA-MB-231 cells were plated on 12-well plates at the density of ... and allowed to reach..... The cells were then rinsed with PBS and serum-free medium, with the indicated concentrations of

CpG-ODNs or vehicle was applied. An equal amount of protein was applied to zymographs (Novex 10 % gelatin gels, Invitrogen, Carlsbad, CA) according to manufacturer's suggestions. In further experiments, aprotinin (2μM) or GM6001 (2μM) were added to the final incubations, to investigate whether CpG-treatment induced serine protease or MMP-activity.

MMP-13 ELISA. MDA-MB-231 cells were plated on 24-well plates at the density of 10^5 cells per well and allowed to reach confluency. The cells were then rinsed with PBS and 200 μl of serum-free medium was added per wells. The supernatants were collected 24 h later and analyzed for levels of active MMP-13 with an ELISA that detects active MMP-13 (Calbiochem, La Jolla, CA), according to the manufacturer's instructions.

Statistical analysis. The results are given as mean ± sd, unless otherwise stated. Student's t test was used to calculate statistically significant differences between the various study groups.

Figure legends

Figure 1. Human breast cancer cell lines exhibit different levels of TLR9 expression. a) The expression profile of the mRNAs for various TLRs was studied with a DNA-array. The calculated levels of expression of each TLR mRNA were obtained after blank subtraction and correction for the expression level of actin. b) Specific expression of the TLR9 protein was detected in permeabilized MDA-MB-231 cells, using PE-conjugated anti-TLR9 antibody in Flow cytometry. c) Western blot detection of the TLR9 protein in the various human breast cancer cells (upper panels). The same blots were stripped and reblotted with anti-actin antibodies, to show equal loading.

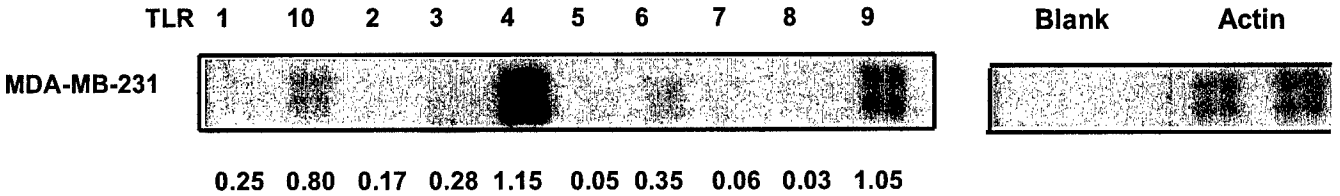
Figure 2. TLR9 agonistic CpG-ODNs induce invasion of TLR9 expressing cancer cells *in vitro*. a) Human breast cancer cells were treated for 24 h with 10 μM CpG-ODNs or with vehicle and the effects on cell viability were tested with MTS-assays. Data represents viability as a percentage of vehicle control, mean ± sd, n=4. b) The effects of CpG-ODNs on the invasive capacity of MDA-MB-231 cells were studied in Matrigel-assays. Data represents the fold-increase in the number of invaded cells, as compared with vehicle controls (dotted line) in each group. Mean ± sd, n=4, * $p< 0.05$, *** $p<0.001$ vs. vehicle. c) MDA-MB-231 cells were cultured for 7 days on 3-dimensional collagen cultures in the presence of vehicle or 10 μM CpG-ODNs. The arrows indicate the front of the invading cells in the gels after they were prepared into H&E-stained histological samples. d) The numbers of invading cells were counted from 5 representative sites in the cut sections. Mean ± sd, n=3, representing the number of sections viewed in each group, * $p< 0.05$ vs. vehicle. e) Western blot detection of the TLR9 protein in U373 astrocytoma and in D54MG glioblastoma cells (upper panels), where MCF-7 cells represent a negative control. The same blots were stripped and reblotted with anti-actin antibodies (lower panels), to show equal loading. f) The effects of CpG-ODNs on the invasive capacity of the indicated cells were studied in Matrigel-assays. Data represents the fold-increase in the number of invaded cells, as compared with vehicle controls (dotted line) in each group. Mean ± sd, n=4, ** $p< 0.01$, *** $p<0.001$ vs. vehicle.

Figure 3. CpG-ODN-treatment increases MMP-activity in MDA-MB-231 cells. a) Supernatants from CpG-ODN-treated MDA-MB-231 cells were run on 10 % gelatin gels. Treatment with CpG-ODNs resulted in the appearance of a gelatinolytic band of ~ 40-50 kDA (arrow), which did not disappear in the presence of aprotinin but which was abolished by the addition of a global MMP-inhibitor, GM6001, to the final incubation. b) The MMP-inhibitor, but not aprotinin (both at 2 μM) inhibited also CpG-ODN-induced invasion. Data represents the number of invaded cells as a percentage of the CpG-induced (10 μM) control for each group. Mean ± sd, n=3, *** $p<0.001$ vs. CpG-ODN-treatment alone.

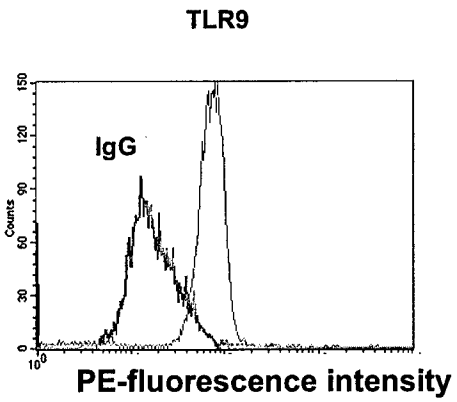
Figure 4. CpG-ODN induced invasion is mediated via MMP-13. a) Levels of active MMP-13 from the supernatants of vehicle or CpG-ODN-treated (10 μM) MDA-MB-231 cells, as analyzed with ELISA. Mean ± sd, n=2, *** $p<0.001$ vs. vehicle. b) The invasive capacity of MDA-MB-231 cells was investigated in Matrigels in the presence of 10 μM CpG-ODNs with neutralizing antibody against MMP-13 or with a control IgG antibody. Data represents the number of invaded cells. Mean ± sd, n=3, ** $p<0.01$ vs. IgG-treated group.

Figure 1.

a.


TLR 1 10 2 3 4 5 6 7 8 9
Blank
Actin
MDA-MB-231
0.25 0.80 0.17 0.28 1.15 0.05 0.35 0.06 0.03 1.05

b.


TLR9
IgG
Counts
PE-fluorescence intensity

c.

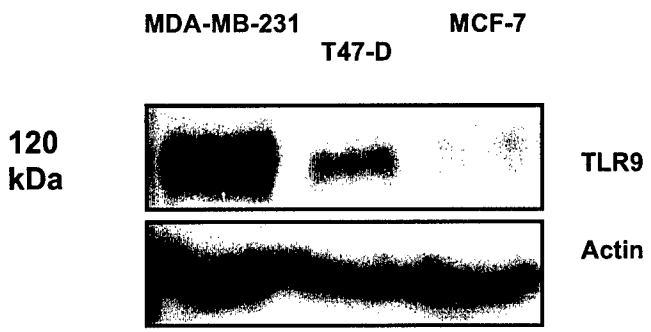
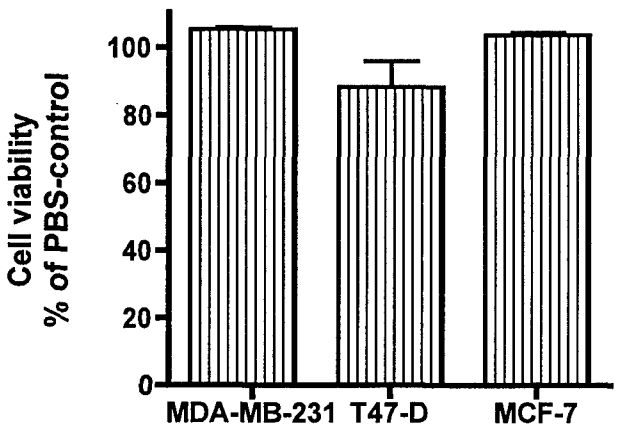
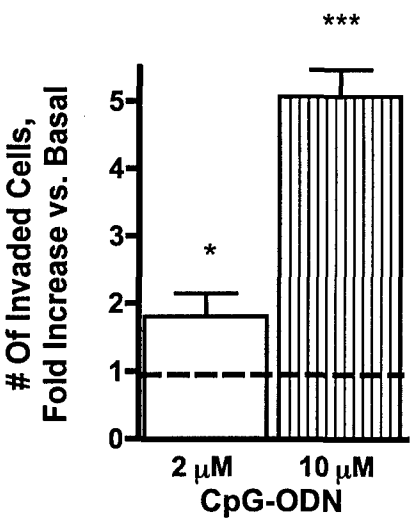

MDA-MB-231
MCF-7
T47-D
120 kDa
TLR9
Actin

Figure 2.

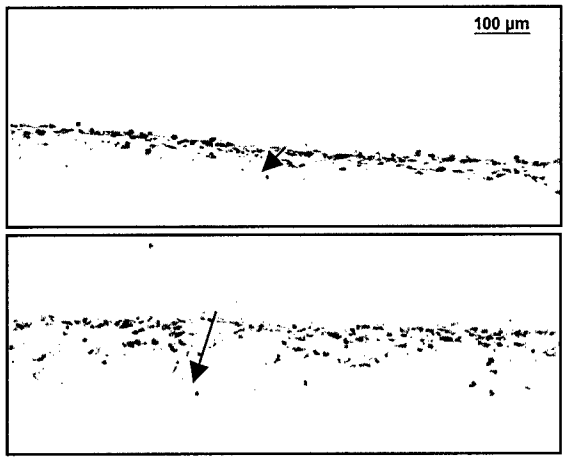
a)


Cell viability
% of PBS-control
100
80
60
40
20
0
MDA-MB-231
T47-D
MCF-7

b)


***
*
Of Invaded Cells,
Fold Increase vs. Basal
5
4
3
2
1
0
2 μM
10 μM
CpG-ODN

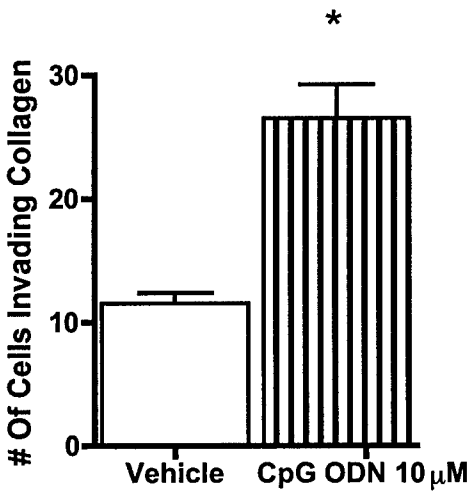
c)



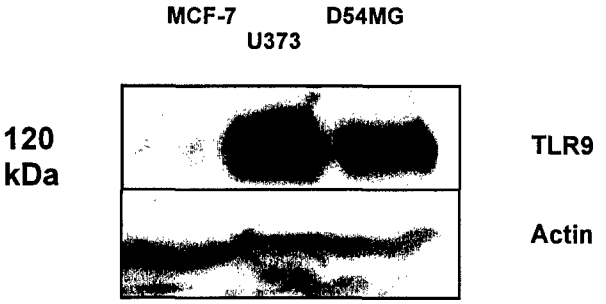
Vehicle

CpG-ODN
10 µM

d)



e)


MCF-7
U373
D54MG
120
kDa
TLR9
Actin

f)

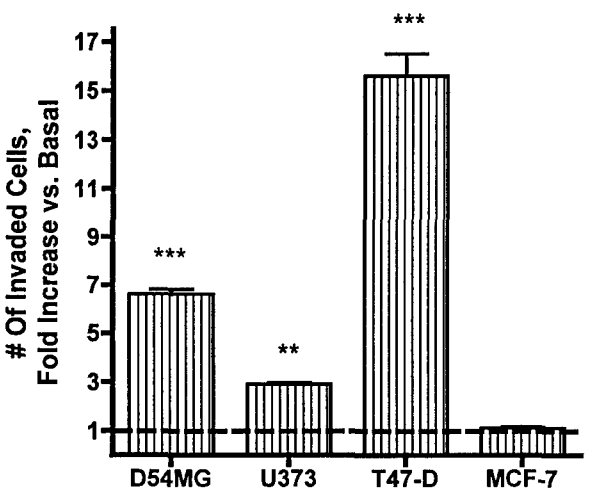
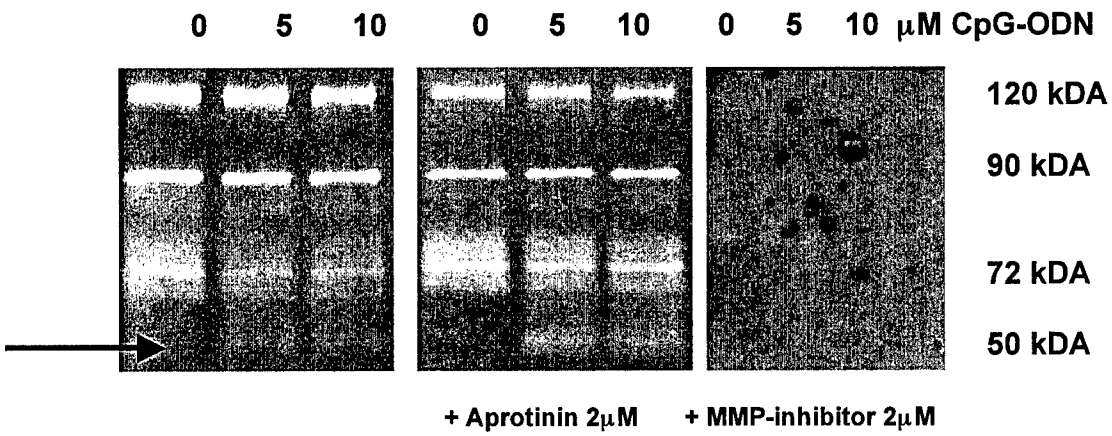

Of Invaded Cells,
Fold Increase vs. Basal
17
15
13
11
9
7
5
3
1
***
**
***
D54MG
U373
T47-D
MCF-7

Figure 3.

a.


0 5 10 0 5 10 0 5 10 μM CpG-ODN
120 kDA
90 kDA
72 kDA
50 kDA
+ Aprotinin 2μM
+ MMP-inhibitor 2μM

b.

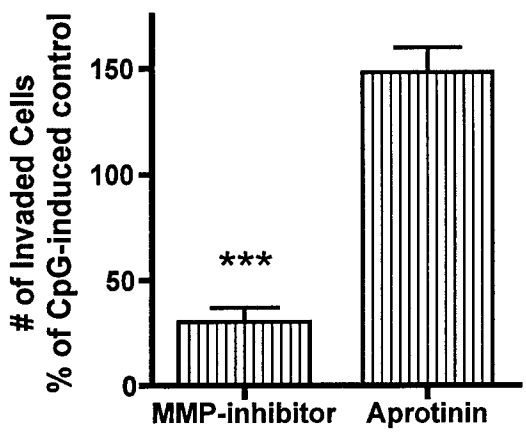
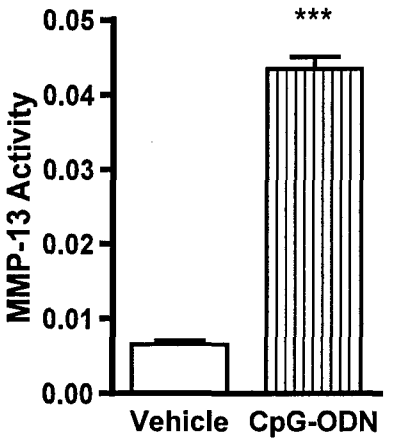
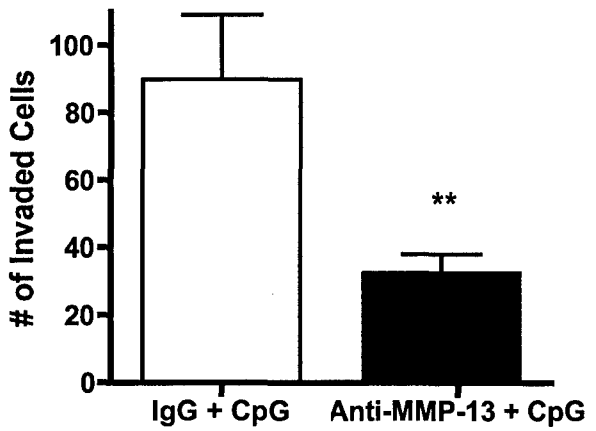

# of Invaded Cells
% of CpG-induced control
150
100
50
0
***
MMP-inhibitor
Aprotinin

Figure 4.

a)


0.05
0.04
0.03
0.02
0.01
0.00
MMP-13 Activity
***
Vehicle
CpG-ODN

b)


100
80
60
40
20
0
# of Invaded Cells
**
IgG + CpG
Anti-MMP-13 + CpG

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