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LESSONS FROM TUMOR AND IMMUNOCOMPETENT CELLS

THE QUANTITATIVE ENGAGEMENT OF LIGAND-RECEPTOR INTERACTIONS MODULATES STOP-AND-GO BEHAVIOUR AS WELL AS PROLIFERATION

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Abstract The four main cell functions, proliferation, apoptosis, differentiation and migration, are tightly regulated by external signals that initiate intracellular signal transduction pathways and determine the cellular behaviour. The concentration and composition of such external signals are at least important for the decision of cells as to which function has to be executed. Interleukin-8 is a well known inducing signal for neutrophil granulocyte migration, while the epidermal growth factor is an inducing signal for breast carcinoma cell migration. Depending on the concentrations of interleukin-8, the neutrophil granulocytes are capable of migration. However, at high concentration of interleukin-8 the migratory activity of each single cell is reduced, indicating that high concentrations of the chemokine inhibit migration and promote the performance of other cell functions. Concerning breast carcinoma cells, the epidermal growth factor is not only an inducer of migration but also an inhibitor of proliferation. These two examples provide evidence for a dose dependent action of external signals for several cell functions in parallel. This versatility of the effects of one ligand might be based on several intracellular signal transduction pathways that are turned on. For the dose-dependent differences of the effect of interleukin-8 we propose a two wheel model of an inositolphosphate-mediated, ATP-independent release of calcium from intracellular stores and a cyclic AMP-mediated, ATP-dependent uptake of calcium into the endoplasmatic reticulum.

Key words: chemokines, chemotaxis, growth substances, oncogenes, protein-tyrosine kinase, cell movement

Resumen ***Enseñanzas de células tumorales e inmunocompetentes. El compromiso quantitativo de las interacciones ligando-receptor modula tanto el comportamiento* stop-and-go *como la proliferación.*** Las cuatro funciones principales de la célula, proliferación, apoptosis, diferenciación y migración, están estrechamente reguladas por señales externas que inician las rutas o vías metabólicas de transducción de señales intracelulares y determinan el comportamiento celular. La concentración y composición de estas señales externas son al menos importantes para que las células decidan qué función deben ejecutar. La interleukina-8 es una muy conocida señal de inducción para la migración de los granulocitos, mientras que el factor de crecimiento epidermal es una señal de inducción para la célula del carcinoma mamario. La capacidad de migración de los granulocitos neutrófilos depende de la concentración de interleukina-8. Sin embargo, frente a altas concentraciones de interleukina-8, la actividad migratoria de cada célula es reducida, indicando que altas concentraciones de esta quimiokina inhiben la migración e inducen otras funciones celulares. En cuanto a las células de carcinoma mamario, el factor de crecimiento epidermal no sólo es un inductor de migración sino también un inhibidor de proliferación. Estos dos ejemplos proveen evidencia para una acción dependiente de la dosis de señales externas para varias funciones celulares en paralelo. Esta versatilidad de los efectos de un ligando podría depender de la activación de varias vías intracelulares de transducción de señales. Para el diferente efecto dosis-dependiente de la interleukina-8, proponemos un modelo de dos ruedas de liberación de calcio de depósitos intracelulares de manera mediada por inositolfosfato e independiente de ATP y captación de calcio en el retículo endoplásmico mediado por AMP cíclico y dependiente de ATP.

Each single cell of a multicellular organism is able to perform four main cell functions (Fig. 1). Proliferation and apoptosis are processes by which the cell number of the organism is regulated. Differentiation and migration are cell functions that are necessary for the maturation of the cell and for the execution of effector functions. The cells are able to switch between these functions. Therefore, the cells have to integrate a plethora of signals and to deduce a proper reaction to these signals. The reaction depends on the intensity of the signals (i.e., the concentration of a ligand or the frequency of ligand/receptor-interactions) and on the

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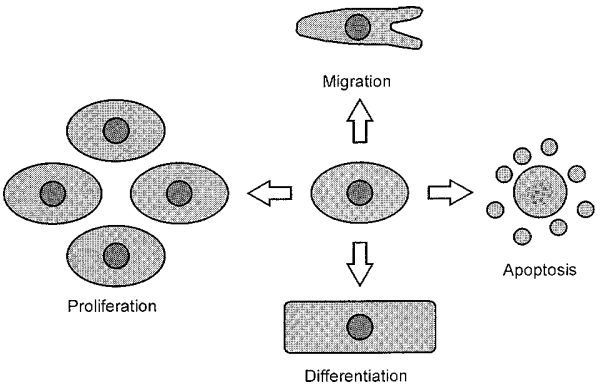

Fig. 1.– The four cell functions

combination with other signals, thus, on the orchestration of signals.

As reviewed recently[1], chemokines and cytokines are inducing signals for cell migration. Herein we show that the concentration of such a migration inducing signal determines distinct reactions of the cells. We furthermore provide evidence that an incoming signal does not cause one particular reaction: the cell functions are not performed exclusively.

Material and Methods

Isolation of human neutrophil granulocytes

Neutrophil granulocytes were isolated from human peripheral blood as described previously[2]. First, a density-gradient centrifugation using Ficoll-Hypaque (ICN, Meckenheim, Germany) was performed. The pellet containing neutrophil granulocytes and erythrocytes was mixed with the platelet-depleted serum and subsequently diluted 1:1.3 with a high molecular weight dextran solution (Makrodex, Fresenius, Bad Homburg, Germany) containing 0.01 M EDTA. After three hours erythrocytes had settled down and the neutrophil granulocyte containing supernatant was separated. Contaminating erythrocytes were removed by hypotonic lysis with 0.3% sodium chloride for 2 minutes on ice. The obtained neutrophil granulocytes contained less than 5% eosinophil granulocytes and T lymphocytes, as was determined by Pappenheim-staining. The cells were used for experiments immediately after isolation.

Cell lines and cell culture

The human breast adenocarcinoma cell line SKBR3 (HTB 30) was purchased from ATCC (American Type Culture Collection, Rockville, MD) and was maintained at 37 °C in the presence of 5% CO_2 in Dulbecco's minimal essential medium (DMEM, LAA Laboratories, Linz, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS, LAA Laboratories, Linz, Austria), 100 units/ml penicillin, and 100 µg streptomycin/ml (Gibco BRL, Eggenstein, Germany). SKBR3 cells exhibit a four- to eightfold amplification of the HER2/neu gene, associated with elevated amounts of $p185^{c\text{-}erbB\text{-}2}$ mRNA and with an overexpression of HER2/neu[3]. The human breast adenocarcinoma cell lines MDA-MB-468-NEO and MDA-MB-468-HER2 were generated by stable transfection of the HER2/neu negative cell line MDA-MB-468 (HTB 132; also purchased from ATCC) with a control vector and the HER2/neu expression vector pCVN-HER2 (kind gift of A. Ullrich, Martinsried). Both cell lines were maintained at 37 °C in Leibowitz 15 (L-15; Gibco BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; Roche Diagnostics, Mannheim, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco BRL, Eggenstein, Germany), and 400 µg/ml G418 (Calbiochem, Bad Soden, Germany).

Time-lapse videomicroscopy and computer-assisted cell tracking

Collagen lattices were generated as previously described[4]. Briefly, 200,000 neutrophil granulocytes or 60,000 tumor cells were mixed with 100 µl of buffered collagen solution (pH 7.4) containing 1.67 mg/ml bovine collagen type I (Invitrogen, Cohesion Technologies, Palo Alto, CA) in minimal essential Eagle's medium (Flow, McLean, VA). The suspension was filled into self-constructed chambers[5] so that the chambers were filled to the half, and were allowed to polymerize for 20 min at 37 °C and 5% CO_2 humidified atmosphere (Fig. 2). After polymerization interleukin-8 (Pharma Biotechnologie Hannover, Germany) was filled into the second half of the chamber at the double of the desired final concentration (2 to 2000 ng/ml) for the experiments with neutrophil granulocytes and 100 ng/ml epidermal growth factor (EGF; Calbiochem, Bad Soden, German) for the experiments with tumor cells. For control experiments, the chambers were filled with medium alone.

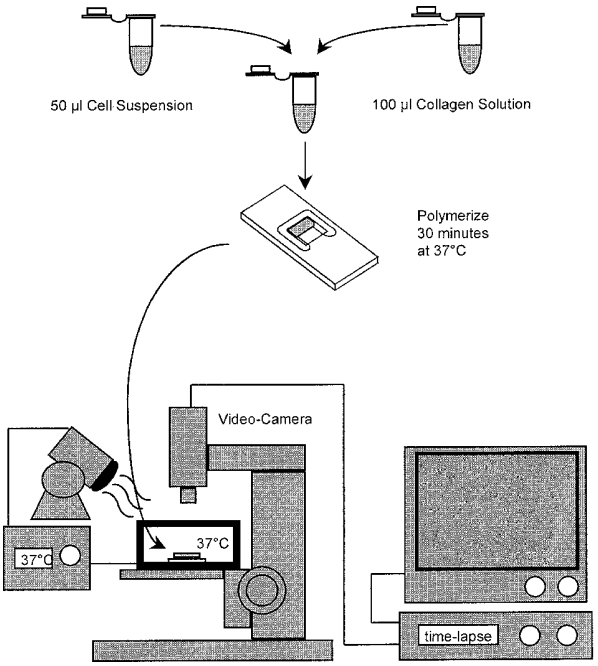

Fig. 2.– Time-lapse videomicroscopy. 50 µl of a neutrophil granulocyte or tumor cell suspension was mixed with 100 µl of a buffered collagen solution and was subsequently filled into self-constructed migration chambers. After polymerization the chamber was sealed and placed under a second chamber which was constantly heated to 37 °C. The locomotory behaviour of the cells was recorded by a time-lapse videorecorder connected to a videocamera.

Migratory behaviour of the cells within the three-dimensional collagen matrix was recorded at a 80 fold time-lapse mode for neutrophil granulocytes or a 1920 fold time-lapse mode for tumor cells, due to the differences in the migration velocity. The temperature of the chambers containing the collagen lattices was continuously kept at 37 °C (Fig. 2). After recording, thirty cells were randomly selected and the paths were digitized by computer-assisted cell tracking. From the digitized paths, the migratory activity of the population was calculated: the migratory activity is represented by the time-average of that part of the observed population (in percent), which was locomotory active at each given time point (one minute intervals for neutrophil granulocytes and 20 minute intervals for tumor cells). In contrast, the displacement (translocating cells in percent) represents, which part of the population was locomotory active within the whole observation period, thus, whether a cell moved or not, regardless how high the locomotory activity of each single-cell was. The locomotory activity of each single cell is at least represented by the time locomoting (in percent): the time locomoting is that part of the observation period, that the cell was actually locomotory active excluding pauses. In addition, the number of cell divisions that were observed within the tumor cell population was counted and calculated as cell divisions in percent of EGF-treated cells compared to untreated control cells.

Flow-cytometry

For the detection of intracellular calcium, the neutrophil granulocytes were resuspended in incubation buffer (10 mM glucose, 140 mM NaCl, 5 mM KCl, 10 mM natrium-HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing the Ca^{2+} indicator fluo-3/AM (1 µM; Molecular Probes Europe BV, Leiden, The Netherlands). The cells were then incubated for 30 minutes at 37 °C. Thereafter the cells were washed twice with fluo-3/AM-free incubation buffer and subjected to flow-cytometry. The mean fluorescence intensity of control cells was adjusted to 350 and changes of intracellular calcium two minutes after addition of 2 to 2000 ng/ml interleukin-8 were measured.

Results

Breast adenocarcinoma cells embedded within a three-dimensional collagen lattice migrate spontaneously

We investigated the migratory behaviour of three breast adenocarcinoma cell lines with different epidermal growth factor receptor (EGFR) and HER2/neu (also named c-erbB-2) expression levels within a three-dimensional collagen lattice. We used the EGFR overexpressing cell line MDA-MB-468-NEO, its HER2/neu overexpressing counterpart MDA-MB-468-HER2[6], and the well established HER2/neu overexpressing cell line SKBR3[3]. After incorporation within a three-dimensional collagen

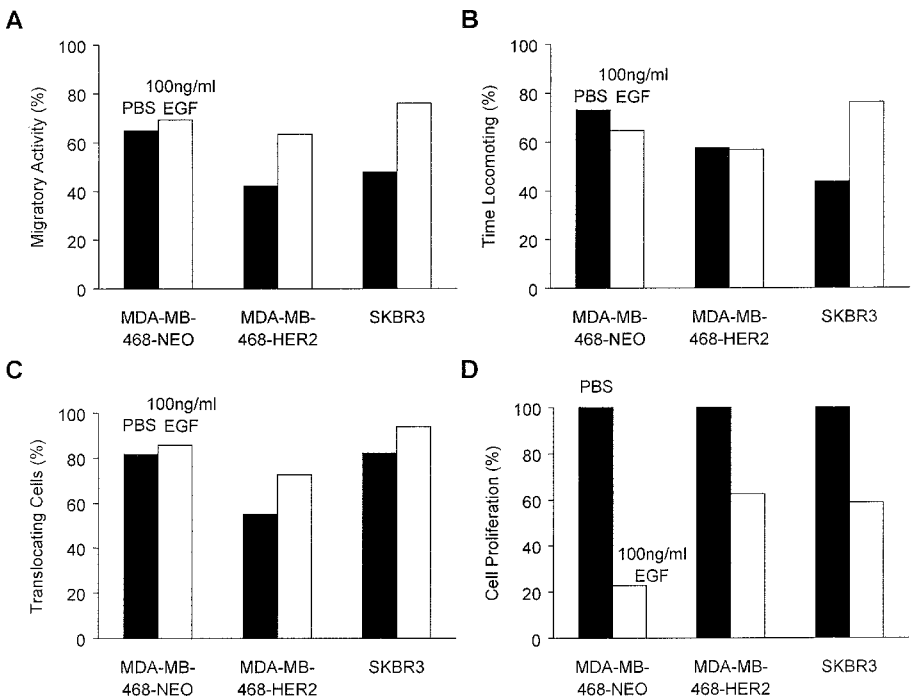

Fig. 3.– Migratory and proliferative activity of breast carcinoma cell lines in response to EGF. 60 000 cells of three different breast adenocarcinoma cell lines (MDA-MB-468-NEO/HER2 and SKBR3) were incorporated within a three-dimensional collagen matrix and treated with either PBS (control) or 100 ng/ml EGF. The migratory activity of the whole population was investigated (A) as well as the locomotory activity on a single cell basis (migratory active time excluding pauses; B) and the part of the population that showed locomotory activity (C). In addition, the number of mitoses within the observation period was counted (D).

matrix the breast cancer cell lines started to migrate spontaneously. The mean migratory activities of the investigated cell lines varied between 65.0% (MDA-MB-468-NEO; EGFR positive – HER2/neu negative), 41.2% (MDA-MB-468-HER2; EGFR + HER2/neu positive), and 48.0% (SKBR3; EGFR + HER2/neu positive; Fig. 3A). Interestingly, the migratory activity of MDA-MB-468-HER2 was lower as compared to the MDA-MB-468-NEO cells. This difference was attributed to the expression of the HER2/neu receptor and, as a consequence of this, to the changes in the expression levels of particular PKC isoforms as was assessed by Western Blot analysis (data not shown).

EGF increases the migratory activity of EGFR and HER2/neu double positive cells but not of single EGFR positive cells

Since tumor cell migration could be stimulated by growth factors[6, 7], we further investigated the migratory behaviour of these cell lines in the presence of the epidermal growth factor. EGF is the ligand for the EGFR and it is known that this growth factor also promotes the dimerization of EGFR and HER2/neu[8]. However, a ligand which promotes HER2/neu homodimerization is still not known. Interestingly, an EGF induced migration could only be observed in the EGFR and HER2/neu double positive cell lines MDA-MB-468-HER2 and SKBR3 but not in the MDA-MB-468-NEO cell line (EGFR positive but HER2/neu negative). The migratory activity of MDA-MB-468-HER2 and SKBR3 cells rose from 42.2% (control cells) up to 63.5% (EGF treated cells), and from 48.0% up to 76.0%, respectively (Fig. 3A). These increased migratory activities of the two cell lines were caused by two different mechanisms. Because the time locomoting of individual MDA-MB-468-HER2 cells was unaffected by EGF (Fig. 3B) we conclude that EGF urges these cells to change their behaviour from a sessile phenotype to a motile phenotye, thus, EGF acts as a start signal for migration (Fig. 3C). In contrast, the time locomotion of EGF treated SKBR3 cells was about 33% higher as compared to untreated SKBR3 cells indicating that EGF prolongs migratory activity (Fig. 3B) and does not only induce migration in non-locomoting cells. The MDA-MB-468-NEO cell line did not react to EGF treatment with an increased migratory activity (Fig. 3A). The slightly higher migratory activity of EGF treated MDA-MB-468-NEO cells was attributed to an EGF mediated survival effect on those cells as verified by propidium iodide staining.

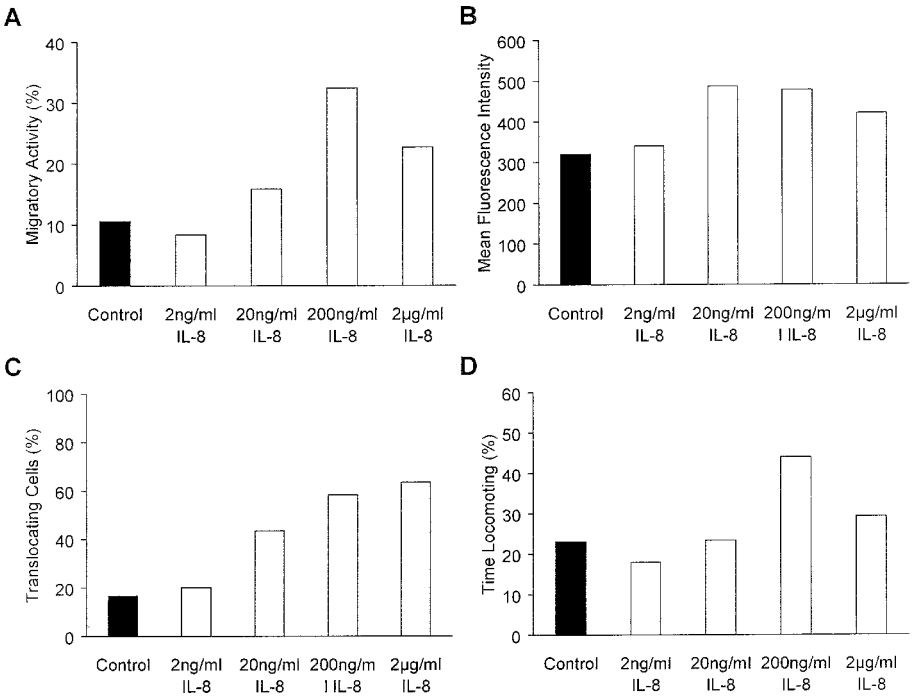

Fig. 4.– Migratory activity of neutrophil granulocytes in response to different concentrations of interleukin-8. A: time-average of the migratory activity of the whole population; B: changes of intracellular calcium in response to increasing concentrations of interleukin-8; C: initiation of migration by interleukin-8; D: migratory activity of each single cell within the observed population (migratory active time excluding pauses).

The proliferation rate of EGF treated breast adenocarcinoma cells embedded within a three-dimensional collagen lattice is markedly decreased

To estimate the effects of EGF on cell proliferation we counted the total number of cell divisions that were observed in all migration experiments. Because EGF is a growth factor not only the locomotion of cells but also the proliferation of cells should be increased. The mean number of cell divisions of EGF treated cell were compared to the number of cell divisions of untreated cells which were set to 100% (Fig. 3D). The interesting finding was, that in all experiments the proliferation rates of EGF treated cells was markedly decreased as compared to untreated cells. The reduction of the cell proliferation rate varied from approximately 40% (MDA-MB-468-HER2 and SKBR3) to nearly 80% (MDA-MB-468-NEO; Fig. 3D).

The effects of interleukin-8 on the migratory activity of neutrophil granulocytes differ depending on its concentration

The induction of migratory activity of human neutrophil granulocytes by interleukin-8 was dose-dependent (Fig. 4A). We observed a bell-shaped curve with a maximum migratory activity of 32.4% at a concentration of 200 ng/ml interlukin-8. Interestingly, the intracellular calcium concentration was elevated by interleukin-8 in the same dose-dependent manner (Fig. 4B). The bell-shaped curve of migratory activity was the net effect of two changes of locomotory behaviour of the neutrophil granulocytes: whereas the concentration of interleukin-8 had a strong, direct correlation to the initiation of migration (translocating cells) even at the highest concentration investigated (2 µg/ml interleukin-8; Fig. 4C), the migratory activity of each single cell (time locomoting) was markedly reduced at 2 µg/ml interleukin-8 (Fig. 4D).

Discussion

Interleukin-8 has distinct effects on the migratory behaviour of neutrophil granulocytes

Interleukin-8 provides a strong start signal for trans-epithelial migration[9] and chemotaxis of neutrophil granulocytes in a dose-dependent way[10]. On the other hand, high concentrations of interleukin-8 reduce the locomotory activity of each single cell. Thus, the time within a certain observation period, that a single cell migrates is lower, or in other words: the time, that the cells stop to migrate (i.e. pauses) is increased. This effect might, at least in part, be based on the intracellular calcium concentration, which shows the same bell shaped curve of dose-dependence as the migratory activity and the time locomoting. For the initiation of migration, an additional pathway might be responsible.

The signal transduction pathways underlying the interleukin-8 effect are mediated into the neutrophil granulocytes by serpentine receptors[11]. These receptors are intracellularly coupled to heterotrimeric G proteins. Activated G proteins induce two distinct pathways: an increase in intracellular calcium is caused by the phospholipase Cβ1 and 2 mediated breakdown of phosphoinositides[12]. On the other hand, an increase of cellular cyclic AMP is mediated by an activation of the G protein dependent adenylyl cyclase. Cyclic AMP has been shown to potentiate fMLP induced migration of neutrophil granulocytes over a very small concentration range and has a strong inhibitory effect at higher concentrations[13]. Cyclic AMP activates the protein kinase A, which in turn terminates the inhibition of calcium ATPases that transport calcium into the endoplasmatic reticulum. Thus, both pathways that are potentially initiated by chemokines are antagonistic on the level of calcium (Fig. 5): a cAMP dependent pathway reduces the cytosolic calcium concentration, whereas a phospholipase Cβ-dependent pathway induces a release of calcium from the endoplasmatic reticulum into the cytosol. We hypothesize that this dichotomy of pathways is responsible for the distinct effects on the migratory behaviour and the bell-shaped curve of migratory activity: at low concentrations of interleukin-8, a strong phospholipase Cβ mediated increase of cytosolic calcium is poorly compensated by the activation of calcium ATPases. At very high concentrations of interleukin-8, a further increase of calcium release from the endoplasmatic reticulum is not possible, but the activity of the calcium ATPases is increased and able to compensate the increment in cytosolic calcium. This regulatory mechanism would explain the migratory activity on a single-cell basis, i.e. the time, each cell actually migrated within the observation period. The initiation of migration might be independent of this calcium regulation. The activation of the phospholipase Cβ leads in addition to the calcium release to an activation of the protein kinase C. Direct activation of the protein kinase C using phorbol esters led to a strong initiation of migration, i.e. an increase of translocating cells to more than the double (data not shown).

What biological function can this dose-dependence of stop-and-go of neutrophil granulocytes have in inflammatory processes? Neutrophil granulocytes are chemotactically attracted to sites of inflammation by chemokines like interleukin-8, which are produced by macrophages and neutrophil granulocytes that are already present at these sites of bacterial contamination. Thus, neutrophil granulocytes migrate towards the highest concentration of interleukin-8. Our results show, that the more cells are recruited to migrate, the higher the interleukin-8 concentration is. In contrast, at very high

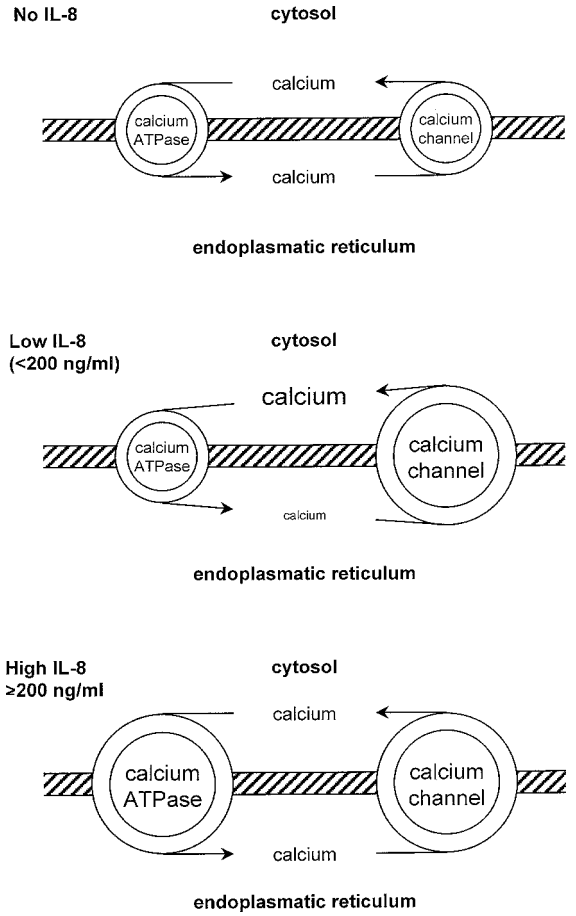

Fig. 5.– Two wheel model of the regulation of cytosolic calcium in neutrophil granulocytes in response to increasing concentrations of interleukin-8. Interleukin-8 activates two signal transduction pathways. Activation of the adenylyl cyclase leads to an increase of cyclic AMP and downstream to an ATP-dependent uptake of cytosolic calcium into the endoplasmatic reticulum. On the other hand, interleukin-8 signaling activates the phospholipase Cβ which in turn produces inositolphosphates. These second messengers cause an ATP-independent release of calcium from intracellular stores.

concentrations interleukin-8 reduces the migratory activity of each single cell. This is a necessary function, since the neutrophil granulocytes have to reduce the migratory activity at the sites of inflammation and have to perform other cell functions (i.e. phagocytotic activity). The recruitment of cells is unaffected by this mechanism. In summary, interleukin-8 acts as a start signal as well as a stop signal for migration of neutrophil granulocytes in dependence of its concentration.

EGF has a promigratory and anti-proliferative effect on breast adenocarcinoma cells within a collagen matrix

Investigating three breast adenocarcinoma cell lines with different EGFR and HER2/neu expression levels we found that the locomotory activity of EGFR and HER2/neu double positive breast cancer cells could be increased by EGF whereas solely EGFR positive cells did not react to EGF treatment with an increased locomotory activity. These results are in accordance with recent findings from Brandt et al. who reported that heterodimerization of EGFR and HER2/neu determines a motogenic phenotype, and, furthermore, only those cells were able to extravasade through an endothelial monolayer which could not be observed with solely EGFR positive cells[6].

Ligand-induced receptor dimerization leads to autophosphorylation of tyrosine residues of the cytosolic domain of the dimerized receptors. Adaptor proteins like Shc (which directs the incoming signal to the Ras-Raf-MAP kinase pathway), p85 (the catalytic subdomain of the phosphoinositol-3-kinase (PI3-kinase)), and the phospholipase-C-γ (PLC-γ) bind to the activated receptor via their SH2 domains and were themselves activated by tyrosine phosphorylation. The role of PI3-kinase in breast adenocarcinoma cell migration remains unclear, but preliminary results indicate that the migratory activity is unaffected by wortmannin treatment (Dittmar and Schewe, unpublished results). The Ras-Raf-MAP kinase pathway directs mitotic signals to the nucleus, whereas activation of the PLC-γ finally leads to protein kinase C (PKC) and gelsolin activation. The activation of gelsolin leads to partial degradation of the actin cytoskeleton which is necessary for cell migration[14]. In addition, activated PKC's translocates to focal adhesin complexes and stabilizes them; a mechanism which also promotes cell migration.

It is known that EGFR/HER2/neu heterodimers are very stable and that most of the incoming signal is transduced via the RAS-RAf-MAP kinase pathway through the nucleus[15]. This could be the reason for the observed effect, that cell proliferation is less decreased in MDA-MB-468-HER2 and SKBR3 cells, because of the strong involvement of the Ras-Raf-MAP kinase pathway. In addition, the stability of the EGFR/HER2/neu heterodimer could be a good explanation of the increased migratory activity because of a prolonged activity of the adaptor protein PLC-γ.

The reduced cell proliferation rate and the unaffected locomotory activity of MDA-MB-468-NEO cells upon EGF treatment could be very well explained by EGFR internalization after EGF stimulation[16] and by EGFR inactivation via threonin phosphorylation of the threonin residue at position 654 via activated PKC's[17]. Due to receptor inactivation, migratory and proliferatory signals were missing.

In summary, both inducers of migration, interleukin-8 for neutrophil granulocytes and EGF for breast adenocarcinoma cells have at least two distinct effects on the cells. For both cell types, we propose that the binding of the ligand to the specific receptor initiates more

than one pathway which leads to different effects (in case of the tumor cells) or even opposite effects (in case of the neutrophil granulocytes). Thus, the amount of receptors occupied by the ligand is a critical parameter for the net effect of cell behaviour.

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J'ai commencé ce livre en tâchant d'expliquer pourquoi notre condition est inexorablement liée a l'imprévisible. A l'impossibilité de répondre à la question qui nous intéresse le plus au monde: que se passera-t-il demain? Ne pas savoir de quoi demain sera fait affecte chacun de nous de manière différente. Il y a ceux qui voudrait savoir s'ils trouveront un emploi, s'ils gagneront aux courses, si leur amant(e) les aimera encore, s'ils seront encore en vie. En ce qui me concerne, ce qui me touche le plus, c'est de ne pas savoir ce que sera ce monde dans cinq cents ans. Ou même cent ans. Ou même vingt ans!

He comenzado este libro intentando explicar por qué nuestra condición va inexorablemente unida a lo imprevisible. A la imposibilidad de responder a la cuestión que más nos interesa en el mundo: ¿qué pasará mañana? No saber de qué estará hecho el mañana afecta a cada uno de manera diferente. Los hay que querrían saber si van a encontrar un empleo, si ganarán a las carreras, si su amante les seguirá amando, o si todavía van a estar vivos. Por lo que a mí me concierne, lo que más me preocupa es no saber qué va a ser del mundo en los próximos quinientos años. O en los próximos cien años, ¡O en los veinte próximos!

François Jacob

La souris, la mouche et l'homme. Paris: Editions Odile Jacob, 1997, p 237