Relevance of tumor microenvironment for progression, therapy and drug development

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Tumor interstitium exhibits a microenvironment that differs from corresponding normal tissues. Tumor phenotype shows, for example, an elevated intracellular pH (pH_i), a lowered extracellular pH (pH_e), a low oxygen concentration and low glucose levels. These differences are caused by cell biological (so called intrinsic) factors, e.g. a higher acidification rate, as well as by more systemic (extrinsic) factors, e.g. poor tumor vascularization. They represent important factors for invasiveness, immune suppression and proliferation, and they imply possibilities for diagnosis, prognosis and therapy. We have developed an experimental data-based computer model, which has simulated the potential role of metabolic effects on tumor progression. We show an experiment on cellular metabolism demonstrating the immunosuppressive impact of low pH_e on peripheral blood mononuclear cells. Finally, we review important findings on the tumor microenvironment leading to possibilities for therapy which are currently evolving and which promise higher effectiveness for cancer therapy. *Anti-Cancer Drugs* 15:7–14 © 2004 Lippincott Williams & Wilkins.

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Introduction

Malignancies of epithelial origin are the dominant cause of cancer death. Their resistance to radiotherapy and chemotherapy is the main reason for ineffective therapy. This lack of responsiveness is partly caused by genetic alterations affecting the signal-transduction machinery of tumor cells. On the other hand, metabolic factors such as pH distribution, tissue oxygen concentration and nutrient supply are found to be important. They have been considered to a much lesser extent in the past, although they have important effects on tumor growth, invasion and responsiveness. At present, it is not clear whether these changes are caused by the cell biological alterations of the tumor (intrinsic) or if they are to be considered as a consequence of extrinsic factors, e.g. bad vascularization. Either way, or caused by both, they represent common findings in tumors and offer possibilities for treatment. It seems feasible that only the combined understanding of the different factors for tumor growth—intrinsic and extrinsic—can lead to a solid understanding and adequate therapy of epithelial neoplasms. Traditionally, only considered in solid tumors, it has been recently demonstrated that microenvironmental changes also affect non-solid hematologic malignancies by protecting them from apoptosis, and promoting tumor cell survival and progression [1].

Causes of microenvironmental changes

Perfusion

Neoplastic tissue growth as well as metabolic and physiological changes in tumors are the reason for highly irregular microvasculature with arterio-venous shunts, blind ends, tortuous innervation and incomplete endothelial lining [2]. This causes perfusion insufficiency with microenvironmental changes such as hypoxia, low glucose levels, low pH values and elevated lactate concentrations. The critical distance between a cell and the nearest blood vessel for oxygen perfusion being 150 μm [3], malperfusion can be a factor for the induction of metabolic alterations and for the necrosis which is commonly found in large tumors. Poor lymphatic drainage leads to fluid accumulation. Triggered by all these changes and mediated mainly by vascular endothelial growth factor (VEGF), vascular growth is stimulated and vascular permeability increased. This in turn results in inhomogeneous vascularization, plasma protein extravasation and replacement of the resulting matrix with vascularized stroma [4–6]. Therefore, it is not surprising that VEGF expression is correlated with proliferation and metastasis of some human cancers [7].

Extracellular pH (pH_e)

Measurements of pH *in vivo* have shown that tumor interstitium is more acidic than normal tissues with

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values for pH_e of 5.6–7.6 [8–10]. With glycolysis as the dominating metabolic pathway, lactic acid is produced even under aerobic conditions [10,11]. Hypoxia, a common finding in tumors, induces a coordinated upregulation of the expression of glycolytic enzymes [12]. The acidification of the cellular environment is further promoted by lack of the microvasculature leading to a reduced buffering capacity and inadequate removal of acidic metabolic products. Therefore, tumor interstitial pH distribution is characterized by spatial inhomogeneity close to microvessels [13,14].

Intracellular pH (pH_i)

Despite the production of lactic acid, the pH_i of tumor cells is near neutrality or even slightly alkaline with values in the range of pH 6.9–7.4 [10]. The result is a reverse pH gradient with $pH_i > pH_e$. The elevation of pH_i appears to be a rather universal signal for the activation of metabolic pathways [15] for cell growth and proliferation [16]. For instance, the rates of protein synthesis, DNA synthesis and RNA synthesis as well as the activity of phosphofructokinase (the rate-limiting enzyme of glycolysis) increase with increasing pH_i within the physiological range. Therefore, tumor cells seem to have a great advantage by maintaining pH_i in this range.

Other ions

Like every change in body pH, the previously described pH gradient causes alterations in the equilibria of other ions. Elevation of total P_i, total Ca^{2+} and Na^+ levels, and a decrease of K^+ and HCO_3^- concentrations have been described [4]. The elevated Na^+/H^+ exchange activity causes an increased import of Na^+, which in turn decreases the Na^+/Ca^{2+} exchange rate. This is a reason for the Ca^{2+} accumulation and for the formation of insoluble calcium phosphate, which is thought to be the reason for tumor calcification [17,18] and alterations of the plasma membrane potential [19,20]. Obviously, this provides a further feedback mechanism which could reinforce aberrations in tumor cell signaling.

The impact of ion concentration changes and the transmembrane potential on each other is not yet clear. Yet, the transmembrane potential falls before mitosis takes place. Tumor cells and other proliferating cells indicate a transmembrane potential between –10 and –30 mV, whereas non-dividing cells show membrane potentials between –70 and –90 mV. Normal cells reach a high potential though as they stop proliferating; cancer cells never elevate their potential to high values. The failure of cancer cells to reach high potentials may be linked to their uncontrolled cell division [21]. It is not clear whether this change only reflects a regulatory mechanism of the cell during proliferation or if this is an essential phenomenon that accompanies malign transformation and a prerequisite for tumor growth [22].

Consequences of changes

Tumor microevolution

Whereas an acidic pH_e inhibits growth and proliferation of normal cells, it has a neutral or even triggering effect on tumor cells.. This can be considered as a result of a pH_e-induced shift towards an acidic pH_i [23,24] in normal cells. A tumor cell's pH_i is nearly invariable because of the intrinsic activation of acid extrusion systems and mitogenic signaling pathways. This makes malignant cells much more resistant to pH_e-induced apoptosis [25–29]. Low pH_e also increases the expression of human multidrug-resistance protein [30], which itself is correlated with an alkaline shift of pH_i [31] and an overexpression of mRNA encoding the Na^+/H^+ exchanger [19]. The glucose-regulated stress response to the tumor-specific microenvironment leads to the induction of a reversible resistance to drugs such as etoposide, doxorubicin, camptothecin and vincristine. This resistance is mediated by a cell-cycle arrest at the G_1 phase. Other mechanisms like the decreased expression of DNA topoisomerase (Topo) IIα for the resistance towards Topo II poisons are also involved [32]. The maintenance of pH gradients between the cytoplasm and vesicular compartments as well appears to be related to therapy resistance [33]. The capability for pH_i maintenance, even in an acidic environment, therefore confers a significant advantage to malignant cell populations protecting them from apoptosis and therapy.

Hypoxia

Hypoxia shows similar effects on apoptosis and resistance, and supports the microevolution of malignant cells. Normally inducing apoptosis, hypoxia has no effect on cells with loss of the p53 tumor suppressor gene [34–35]. As with other mutations, loss of p53 is mainly found in cells under physiological stress as, for example, in tumors with low pH_e [36–38]. This implicates that hypoxia acts against apoptosis-competent cells in tumors, thus promoting the clonal expansion of mutant cells [34]. This hypoxia-mediated selection of cells with diminished apoptotic potential might be an important factor for the resistance of many solid tumors to cancer therapy [39–41].

In addition, as a function of oxygen levels, the proliferation rate of tumor cells decreases with the distance from a blood vessel [42]. These cells which rest in the G_1 phase have a temporary resistance against cytostatic drugs, which act only against rapidly dividing cells [43]. The distant parts of tumors are, for reasons of diffusion, exposed to lower concentrations of cytostatics and, due to hypoxia, are less sensitive to radiation therapy [44]. Through these mechanisms they have much higher chances to escape tumor therapy and to establish resistance.

Tumor metabolism

The increased glycolytic metabolism of tumor cells creates an elevated demand for glucose which is provided by an increased number of glucose transporters [45]. This elevated uptake lowers the interstitial glucose concentration and thereby deprives the normal tissue of its energy supply. A competition-theory-based model proposed by Gatenby [46] suggests that the metabolic changes associated with transformation manipulate the tumor microenvironment in such a way that conditions favor growth of tumor cells at the expense of normal cells. In a more recent model it was shown, that although significant spatial gradients of glucose formed, no regions of detrimentally poor glucose developed [47].

Suppression of immune response

Acidic pH_e is a dominant immuno-suppressive factor in solid tumors. It acts as an inhibitor of a proliferative response of lymphocytes [48] and of the release of perforin [49,50]. It lowers the cytotoxic activity of lymphocytes, natural killer cells and human lymphokine-activated killer (LAK) cells towards tumor cells [51–53]. This might explain the accumulation of lymphocytes at a tumor site without obvious cytotoxic effects. Using quantitative fluorescence microscopy to measure alterations of intracellular pH and Ca^{2+} it was demonstrated that the programming time for the killing process by human natural killer cells is shortest at a slightly alkaline pH_e of 7.3–7.6 [54]. This implies the importance of a neutral or slightly alkaline pH_e for a successful immune response.

Invasion

Tumor cell invasion and migration seems to be increased by an acidic environment. Human melanoma cells, for example, which were cultured in acidic conditions became much more invasive [55]. This behavior is supported by the finding that extracellular acidification promotes the secretion of matrix-degrading collagenases [23], the redistribution of cathepsin B to the surface of malignant cells, its secretion and its activation [56,57]. Moreover, the chemotactic migration of tumor cells through type IV collagen-coated membranes [23] is enhanced in an acidic medium. In breast cancer cells (MDA-MB-231), the secretion of connective tissue growth factor, a potent angiogenic factor contributing to the invasion of breast cancer cells, is upregulated by hypoxia [58].

Metastasis

Some authors also mention microenvironment, especially hypoxia, as a factor that promotes metastasis. Mechanisms like DNA damage caused by hypoxia and following re-oxygenation, selection of metastatic cell phenotype by physiological pressure or induction of gene products for the metastatic cascade seem to be the cause [59].

Tumor re-oxygenation during radiation therapy may promote microenvironment-induced metastasis by rescuing hypoxic or nutritionally deprived metastatic cells from dying. Ionizing radiation can elicit a stress response in tumor cells similar to that elicited by hypoxia. Radiation therapy may therefore adversely affect the rate of metastasis in patients who do not achieve control of the primary tumor by enhancing the expression of gene products of importance in metastasis [59].

Approaches to a dynamic analysis of microenvironmental changes

We have outlined the complexity of cellular interactions that influence tumor microevolution and response to therapy. Obviously, the depicted framework is too complicated for *in vivo* analysis, an intuitive understanding or even a prediction of systems behavior. The main reason is that cellular systems function as a nonlinear and complex signaling network. In addition to the 'conventional' genetic and biochemical approaches, valuable tools for the analysis of tumor microevolution have been developed. These are computer-based modeling, sensor-assisted techniques and *in vitro* models, which even complement each other.

Computer simulations for the analysis of tumor progression

Computer simulation in biology is a rapidly growing field that encompasses the theory and application of computational approaches to model, predict and explain biological function at the cellular or even molecular level [60]. Enabled by suitable tools for systems analysis, increasing computer power and appropriate algorithms, even large systems can be investigated by means of computer simulations. Such simulations can be manipulated to explore competing hypotheses about tumor microevolution, invasion and immune response, i.e. used as a computer-based experimental system.

To investigate tumor microevolution, a series of computer models has been constructed by the authors and others [46,61,62]. These models have served as a framework by which experiments can be performed '*in machina*'. Relationships between the various parameters considered have been established. The models support a 'minimal scenario' for tumor microevolution which, in brief, encompasses the following mechanisms. Induced by mitogenic stimulation and/or oncogenic alterations in their signal transduction network, tumor cells develop an improved capability for acid extrusion. This clamps pH_i to slightly alkaline values permissive for growth and proliferation, and provides tumor cells with an enhanced resistance against acidic extracellular conditions. Since rapid tumor cell growth under hypoxic conditions is accompanied by glycolytic metabolism, acid production and acid export is further enhanced. Local extracellular

acidification is facilitated by microcirculatory inadequacy resulting in both reduced buffering capacity and functional heterogeneity inside the tumor. Significant microenvironmental pH gradients promote tumor cell invasion and inhibit the immune response. Hence, the acidic microenvironment found in most solid tumors appears to be a main regulator for the self-organized development of neoplastic growth and invasion.

In a different modeling framework, Webb *et al.* have focused on the affects of low tumor pH on invasiveness. Knowing that the incubation of tumor cells at low pH induces more aggressive invasive behavior *in vitro*, the authors examined whether altered proteolytic activity at low pH is responsible for the stimulation of a more metastatic phenotype. The modeling suggests that changes in metalloproteinase (MMP)-activity at low pH do not have significant effects on invasive behavior, whereas the levels of active cathepsin B are significantly altered by acidic pH. This suggests a critical role for this cysteine proteinase in tumor progression [63].

In summary, available computer-based approaches to tumor microevolution support the hypothesis that the changes of the microenvironment by malignant cells towards hypoxia and acidification cannot only be regarded as a supplementary side-effect of tumor cell metabolism. It is rather a strategic principle, most likely involving mechanisms of self-organization [64] to 'escape' processes normally counteracting neoplastic growth and invasion. As summarized above, a solid understanding of the key parameters involved in these processes offers new possibilities for tumor-selective therapy schemes.

In vitro approaches to the analysis of impaired immune function

Motivated by our mathematical modeling and based on own experiments on the inhibition of immune function by modulation of the microenvironment, we studied the effect of a downshift of pH_e on the metabolism of peripheral blood mononuclear cells (PBMCs). PBMCs were used as an example for immune cells. We used a multi-microsensor system developed in our laboratory which allows the on-line registration of cellular respiration by the use of miniaturized sensors for the measurement of oxygen. The cells are nourished by a fluid perfusion system which permits us to change culture media or to apply agents through the continuously replaced culture media. The oxygen concentration of the culture media is continuously measured and changes can be directly attributed to applied agents. It offers a platform for chemosensitivity testing and drug screening. The system and its possible applications have been variously discussed in other publications [65,66], and a more explicit description can be found there. The system can be regarded as a tool to verify our '*in silico*' approach. Monocyte-depleted PBMCs were collected from fresh human donor blood and isolated by Ficoll-Hypaque gradient density centrifugation. The cells were cultured for 3 days and stimulated with interleukin-2 to ensure growth of LAK cells. Then they were connected to the fluid perfusion system and recordings of oxygen concentration in the culture media were started. At the times indicated, normal RPMI culture medium was replaced by media with a lower pH (6.7 and 6.0). A control culture was left in culture medium at pH 7.3. At the end all cells were killed with Triton X-100 to obtain the base signal (Fig. 1).

The measurement (Fig. 2) shows a clear downshift of cellular oxygen consumption and can be interpreted as an inhibition of cellular metabolism in reaction to the acidification of the cellular microenvironment. This correlates with other data on immune suppression of PBMCs and LAK cells under such conditions [51]. The experiment can be regarded as an *in vitro* model for an acidic tumor microenvironment and points out its immunosuppressive effect.

Opportunities for therapy

Microenvironmental changes strongly affect cancer treatment. For example, resistance towards chemotherapy and the success of radiation therapy have been reported to depend on the tumor microenvironment. However, these primarily local alterations offer possibilities for selective cancer treatment. Local microenvironmental changes are confined to areas of malignant growth and can offer a way to confine the action of drugs to these areas, thereby making local and selective chemotherapy possible. New methods allow us to measure the microenvironment *in vivo* and thereby facilitate pre-therapeutic planning.

Using fluorinated vitamin B6 derivatives (6-fluoropyridoxol and 6-fluoropyridoxamine) it is possible to simultaneously measure intra- and extracellular pH *in vivo* by a non-destructive and non-invasive method using nuclear magnetic resonance. Thereby, an exact planning of therapy seems possible [67]. *In vivo* measurement of tissue oxygenation is possible by techniques such as low-frequency electron paramagnetic resonance spectroscopy or dynamic fluorescence quenching [68,69].

Drug ionization

Depending on their ionization potential, weakly acidic drugs that are lipid soluble in their non-ionized state freely diffuse across the cell membrane, and, having entered a relatively basic intracellular compartment, become trapped and accumulate within the cell. Using the reversed pH gradient, this may lead to substantial (10-fold or more) differences in the intracellular-to-extracellular drug distribution between tumor and normal tissue for cytotoxics, hypoxic cell sensitizers or other

Fig. 1

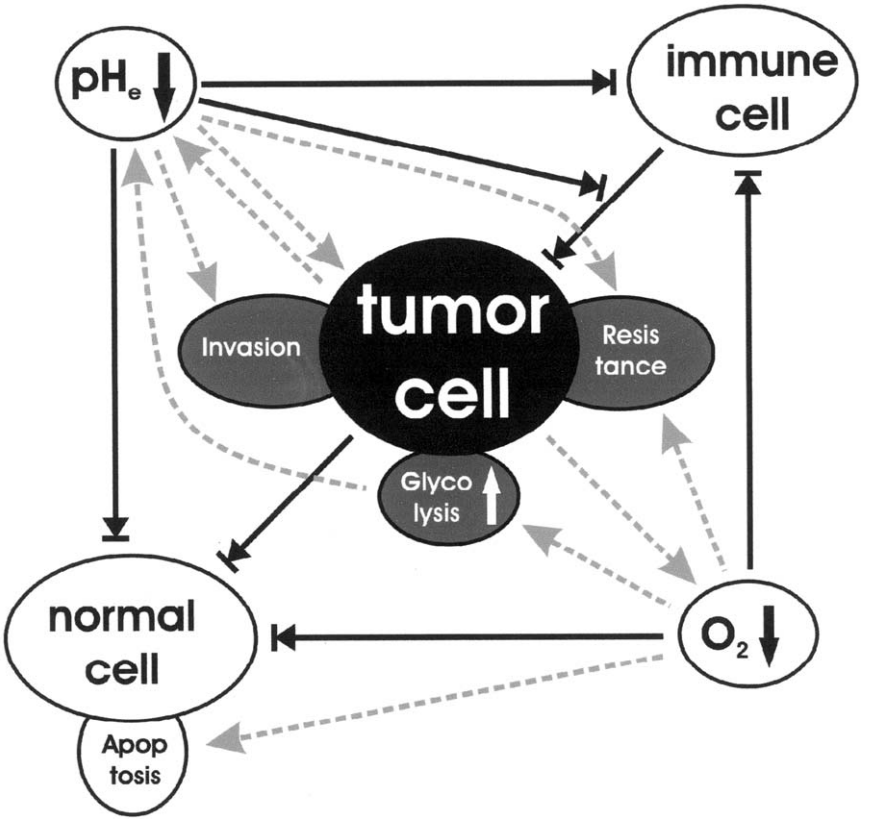


Illustration of some consequences of microenvironmental changes towards tumor cells, normal tissue cells and the immune reaction as mentioned in the text. Solid arrows signify inhibiting effects, dashed arrows signify stimulation towards the subject they are pointing to.

drugs exhibiting the appropriate pK_a in the range of 4.5–6.5 [70]. Experimental *in vitro* evaluation of these predictions confirms both the predicted pH gradient-dependent changes in cellular drug accumulation and toxicity [70]. For example, an increase of the reversed pH gradient across the cell plasma membrane caused a parallel increase in tumor growth delay by the weak acid chlorambucil. This indicates that the changed transmembrane pH gradient is a major and exploitable determinant of the uptake of weak acidic chemotherapeutics, especially effective in combination with radiation [71].

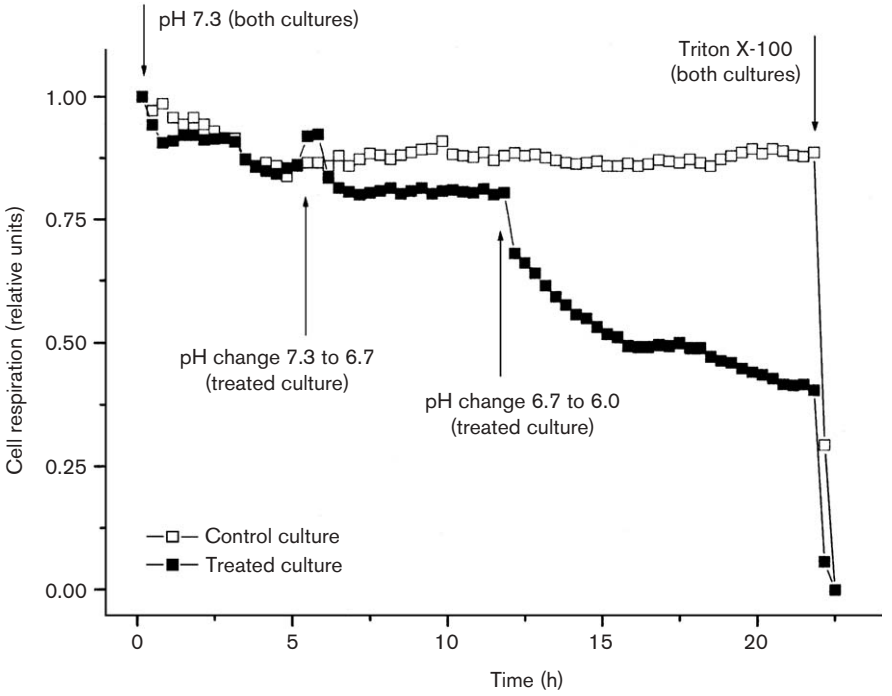
Cells within the acidic microenvironment of solid tumors maintain their intracellular pH through the activity of membrane-based ion-exchange mechanisms. Inhibition of these regulatory mechanisms has been studied as an approach to tumor therapy. Cariporide, an inhibitor of the Na^+/H^+ antiporter, and S3705, an inhibitor of the Na^+-dependent Cl^-/HCO_3^- exchanger are two such agents. Results indicate that cariporide and S3705 function as selective cytostatic agents under *in vitro* conditions [72].

The difference in the ability for regulation of pH_i between tumors and normal tissues might be exploited therapeutically by the disruption of the mechanisms which regulate pH_i, so that tumor cells are killed by intracellular acid-induced injury. Studies showed that acid-induced cell death depends on the apoptosis-related marker Bax and on stress-activated protein kinases (SAPK) signaling pathways, but not on the caspase proteases. Therapeutic manipulation of Bax and SAPK may enhance acid-induced tumor cell killing [73].

Hypoxia-selective cytotoxins

Hypoxia-selective cytotoxins take advantage of the uniquely low oxygen tension in the majority of human solid tumors. Their activation by reduction at low oxygen levels creates two advantages. First, they are tumor specific as hypoxia is relatively tumor specific [74]. Second, it is based on the principle of complementary cytotoxicity, meaning that the cells attacked by hypoxia-selective treatment are the ones resistant to conventional therapy.

Fig. 2



Experiment with PBMCs showing the immunosuppressive effect of low pH_e. At the times indicated the pH of the culture media was lowered and the cellular oxygen consumption dropped as a consequence.

Quinone antibiotics induce the formation of DNA interstrand cross-links when converted on reductive metabolism. They show different specificity for hypoxic regions. Mitomycin is cytotoxic in aerobic and hypoxic conditions [75], and gave good results in combination with radiotherapy of head and neck cancer [76]. The newer agent porfiromycin shows higher specificity for low oxygen levels. Tirapazamine (TPZ) has demonstrated activity in cancer clinical trials. Under hypoxic conditions TPZ is reduced to a radical that leads to breakage of DNA double and single strands, to base damage, and is implicated as a Topo II poison [77]. It has shown excellent efficacy in preclinical trials and maintains its activity at oxygen levels up to 10-fold higher than other hypoxic cytotoxics, thereby attacking intermediately hypoxic cells as well.

Another promising possibility seems to be an approach using gene therapy activated by the low oxygen environment. Transfection of a human fibrosarcoma cell line with a hypoxia-regulated expression vector conferred sensitivity to the prodrug RSU1069 *in vitro*. Xenografts showed this to be a curative regimen [78].

Suppression of the immune response

Local immunosuppression may explain the failure of an effective immune response against solid tumors and is reflected by cytotoxic activity. The suppression of human non-major histocompatibility complex-restricted cytotoxicity against tumor cells by an acidic pH_e was measured using unstimulated PBMCs, LAK cells and natural killer cell clones as effector cells, and target cell lysis was measured. The cytotoxic activity of unstimulated PBMCs and LAK cells was markedly reduced by a decreasing pH_e, and the lytic potential of homogeneous natural killer cell clones as effectors was also impaired. In conclusion, this may contribute to the failure of immunosurveillance against solid tumors. Consequently, efforts to enhance the anti-tumoral cytotoxicity by immunotherapies may have limited success [79].

Microvasculature

Leaky tumor blood vessels can be exploited using liposomes that have been sterically stabilized to have a long intravascular half-time, allowing them to selectively accumulate in solid tumors. Studies have been conducted encapsulating doxorubicin, but distribution of the drug was highly heterogeneous [80]. The idea that tumor growth depends upon angiogenesis and thus that inhibition of angiogenesis could be a powerful anticancer therapy has been thoroughly studied. Inhibitor molecules for the VEGF receptor Flk-1 inhibit (*in vitro* or *in vivo*) angiogenesis, inhibit tumor growth and prevent metastatic spread [81,82]. A recent study using a murine

model showed that cancer gene therapy by the intramuscular delivery of plasmid DNA encoding vasostatin, a potent angiogenesis inhibitor, is effective in the inhibition of the systemic angiogenesis and tumor growth [83].

Outlook

There are various differences in microenvironment between normal and tumor tissue. Reviewing basic facts and recent studies, we have given a summary of what we believe to be the most important alterations. Our own experiments on the immunosuppression of LAK cells by a low pH_e and the results by mathematical modeling of tumor growth are coherent with the presented recent publications. These findings are the basis for new methods in tumor therapy. Functional metabolic imaging/adjusted radiation therapy is considered as one of the main fields of interest in the near future. Tumor-specific alterations which have formerly been considered as factors for tumor progression, malignancy and, most importantly, obstacles for therapy can possibly be exploited. The new plan of action of distinguishing neoplastic cells from normal tissue by differences in their microenvironment may be a novel opportunity for selective therapy.

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