Chapter 11
Carbonic Anhydrase IX: Regulation and Role in Cancer

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Abstract Tumor microenvironment substantially influences the process of tumorigenesis. In many solid tumors, imbalance between the demand of rapidly proliferating cancer cells and the capabilities of the vascular system generates areas with insufficient oxygen supply. In response to tumor hypoxia, cancer cells modulate their gene expression pattern to match the requirements of the altered microenvironment. One of the most significant adaptations to this milieu is the shift towards anaerobic glycolysis to keep up the energy demands. This oncogenic metabolism is often maintained also in aerobic cells. Lactic acid, its metabolic end-product, accumulates hand-in-hand with carbon dioxide, leading to acidification of the extracellular environment. Carbonic anhydrase IX (CA IX) is the most widely expressed gene in response to hypoxia. Its crucial role in intracellular pH maintenance represents the means by which cancer cells adapt to the toxic conditions of the extracellular milieu. Furthermore, the activity of CA IX stimulates the migratory pathways of cancer cells and is connected with the increase of the aggressive/invasive phenotype of tumors. CA IX expression in many types of tumors indicates its relevance as a general marker of tumor hypoxia. Moreover, its expression is closely related to prognosis of the clinical outcome in several tumor types. All above mentioned facts support the strong position of CA IX as a potential drug therapy target. Here, we summarize the state-of-the-art knowledge on its regulation and role in cancer development.

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1 Cancer and Tumor Microenvironment

Solid tumors are characterized by distinct attributes that directly lead to massive changes both in genotype and phenotype of cancer cells. First, mutations-driven uncontrolled cell growth and proliferation support spatial de-organization and a rapid increase of the tumor mass. As the tumor growth advances, areas with insufficient oxygen supply arise due to inadequate vasculature [1]. *Tumor hypoxia*, a condition defined by less than 5 % oxygen in the surrounding tissue, conducts dramatic alterations in cancer cell behavior including changes in gene expression, metabolism, genetic stability, proliferation and survival [1–3]. To address the limited availability of oxygen, the cancer cells shift their energetic metabolism from oxidative phosphorylation towards anaerobic glycolysis [4, 5]. Glycolytic metabolism generates large amounts of lactic acid and carbon dioxide that contribute to a decrease of the extracellular pH (pH_e), leading to the development of acidic milieu within the tumor microenvironment [6]. As the maintenance of vital biological functions of the cell is extremely sensitive to intracellular pH (pH_i) consistency, the survival of cancer cells is dependent on their ability to adapt to these limiting conditions [6]. One of the key players involved in the process of adaptation is carbonic anhydrase IX (CA IX) protein.

2 Carbonic Anhydrase IX: Introduction

CA IX is a transmembrane zinc metalloenzyme functioning as the catalyst of reversible hydration of carbon dioxide to bicarbonate ions and protons [7]. The protein belongs to the α class of the family of carbonic anhydrases (CAs) expressed in higher vertebrates [8]. CA IX (initially denominated "MN antigen") was discovered as a cell surface protein on HeLa cervical cancer cells using monoclonal M75 antibody [9, 10]. Following its identification, the cDNA and genomic sequences of the protein were analyzed, observing the presence of a 257 amino acid (aa) long catalytic domain specific for the family of CAs [11, 12]. Hence, the protein joined the family of carbonic anhydrases as the ninth member and was therefore renamed to CA IX.

3 Biochemical Structure of CA IX

The *CA9* gene is located on chromosome 9p 12–13 and encodes a 459 aa residues long transmembrane protein [13]. The N-terminal extracellular domain (ECD, 414 aa) comprises: (i) 37 aa signal peptide; (ii) 59 aa long proteoglycan-like (PG)

domain bearing features homological to that of keratan sulfate attachment domain of proteoglycan aggrecan; and finally, (iii) the 257 aa catalytic (CA) domain [12]. The presence of the PG domain is unique to CA IX among the CA family. The M75 antibody recognizes a linear epitope within the very PG domain, guaranteeing that the antibody binds CA IX exclusively [14].

The protein spans the plasma membrane by a 20 aa long hydrophobic transmembrane (TM) domain helix and the C-terminal domain of CA IX is protruding into the cytosol in the form of the 25 aa long intracellular (IC) tail [11, 12]. In addition, *CA9* mRNA undergoes alternative splicing (AS) generating the CA IX AS isoform lacking the IC domain, TM domain and a part of the CA domain, due to a frameshift-generated STOP codon at position 1,119 bp [15]. The truncated CA IX AS isoform was observed in cancer cells as well as in normal cells, regardless of the hypoxic conditions [15, 16].

Mature CA IX is represented by a 54/58 kDa double band on western blots in reducing conditions. In non-reducing conditions, a 153 kDa band was observed, possibly corresponding to CA IX homotrimer [9]. Subsequent analyses of the biochemical structure of the protein were carried out in vitro on recombinant CA IX forms lacking the IC tail and TM domain, expressed using baculovirus expression system. Intriguingly, mass spectrometry results suggested the presence of intermolecular S-S bridge occurring between Cys^{41} residues, indicating towards a homodimeric, rather than homotrimeric state of CA IX [7, 17]. The plausible CA IX homodimer might be furthermore stabilized by a number of non-covalent interactions involving two hydrogen bonds and additional van der Waals bonds [7, 18]. Additionally, intramolecular stabilization by disulfide bridge between Cys^{119} and Cys^{299} was identified [7]. Finally, N- and O-linked glycosylations were identified at Asn^{309} and Thr^{78} residues, respectively [7].

4 Catalytic Activity of CA IX

Members of the family of CAs catalyze the hydration of carbon dioxide to bicarbonate ions and protons. This is achieved by deprotonation of a water molecule that is bound to the active site present in the CA domain. To investigate the kinetic properties of CA IX, the aforementioned recombinant CA IX forms expressed in Sf9 insect cells, along with recombinant CA IX from *E.coli* expression system were analyzed using stopped-flow spectrophotometry [7]. Upon comparison with kinetic properties of other mammalian CAs, CA IX and CA II were found to be the most active [7, 19]. The optimal pH value for this process was observed around pH 6.49, compared to the optimal values close to pH 7, which are typical for other CA isoenzymes [19]. Experiments with ΔCA and ΔPG CA IX isoforms revealed that the CA domain alone is sufficient for the catalytic activity of CA IX although a possibility of cooperation with the PG domain in the function of CA IX is not excluded [20]. Interestingly, the unique PG domain might function as an intrinsic buffer, rendering CA IX a suitable pH_i regulator in the conditions of environmental acidosis [19].

The activity of CA IX addresses the rising requirement of the cell to neutralize its pH_i in an acidic environment. Although the expression of the protein is connected with several physiological processes including lipo- and ureagenesis, gluconeogenesis and bone resorption, its most prominent role is connected with pH_i regulation in cancer cells exposed to acidic environment caused by metabolic products of glycolysis [7, 21, 22].

Oncogenic metabolism in highly proliferative cancer cells generates excessive amounts of lactate, carbon dioxide and protons. To maintain optimal pH_i, these metabolites are extruded to the extracellular milieu where they accumulate due to poor vasculature [23]. Due to the continuously decreasing pH_e, cancer cells need to employ another adaptive mechanism. To neutralize their pH_i, bicarbonate anions generated by CA IX-catalyzed hydration of carbon dioxide are transported into the cells by anion transporters to interact with protons liberated by the glycolytic metabolism (described in more detail later in this chapter) [24, 25]. The process generates carbon dioxide, which diffuses through the plasma membrane [26].

CA IX catalytic activity thus contributes to (i) extracellular acidification by elevating the extracellular levels of carbon dioxide and protons; (ii) intracellular neutralization by generating bicarbonate ions, as well as by active participation in the process of their intake by a mechanism described later in this chapter.

Remarkably, a detailed analysis of CA IX interactome revealed an additional, hitherto unanticipated role of CA IX. Among the identified interacting partners, a substantial part was represented by proteins facilitating nucleo-cytoplasmic transport including importin TNPO1 and exportin XPO1 [27]. This finding suggested nuclear localization of CA IX, a notion which was subsequently confirmed by confocal immunofluorescence assay on various cell lines. It seems plausible that the nuclear moiety of CA IX functions by interaction with partner proteins rather than by direct binding to DNA [27]. Although the exact role of nuclear CA IX awaits its identification, these findings imply that the activity of CA IX goes far beyond its catalytic function.

5 CA IX Regulation

5.1 Regulation of CA IX Expression

Transcriptional activity of CA IX is subject to numerous regulatory elements employing various mechanisms of action. However, the principal regulator of CA IX expression is hypoxia via its executive officer, hypoxia-induced factor 1 (HIF-1) [28].

HIF-1 is a transcription factor originally identified as a *trans*-acting component mediating erythropoietin expression in hypoxia [29, 30]. The active transcription factor is a heterodimer composed of oxygen sensitive subunit α (HIF-1α) and constitutively expressed subunit β (HIF-1β) [31]. In normoxia, the fate of HIF-1α

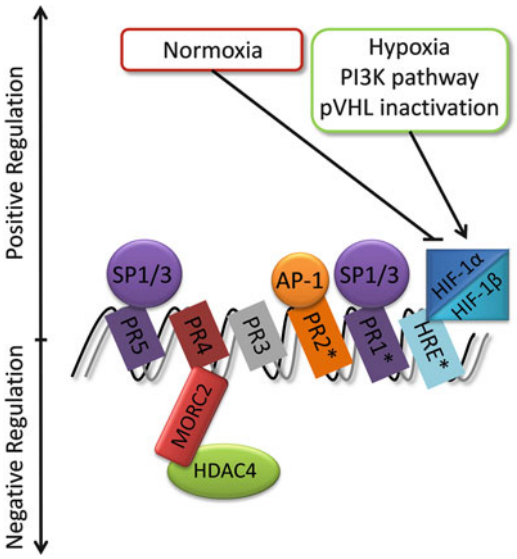

Fig. 11.1 *CA9* promoter regulation. Six distinct *cis* elements were identified within the *CA9* promoter sequence; the hypoxia responsive element (HRE) and five regions identified by DNaseI footprinting (Protected regions, PRs). The HRE element is recognized by HIF-1 heterodimer and its binding is crucial for the *CA9* promoter activity. PR1 and PR5 regions are recognized by the Specificity protein (SP) 1 and/or SP3; PR2 is bound by the Activator protein 1 (AP-1). Regions essential for the *CA9* promoter activity in hypoxic conditions are marked with an asterisk (*). The PR4 region acts as a repressor of the *CA9* promoter upon binding of the Microrchidia2 (MORC2) and Histone deacetylase 4 (HDAC4) complex

subunit is sentenced by von Hippel-Lindau tumor suppressor protein (pVHL)-mediated polyubiquitylation and subsequent proteasome degradation [32]. The process is dependent on pVHL recognition of hydroxylated proline residues Pro^{402} and Pro^{564} located in the oxygen-dependent degradation domain (ODDD) of HIF-1α. Both proline residues are hydroxylated by prolyl-hydroxylase domain proteins (PHDs) only in normoxia. In addition, HIF-1α activity is guarded by asparaginyl hydroxylase enzyme referred to as 'factor inhibiting HIF-1' (FIH-1) [33]. FIH-1 hydroxylates Asn^{803} present in the C-terminal activation domain of HIF-1α and thus prevents it from binding to its co-activators [34]. In low levels of oxygen, PHDs, as well as FIH-1 are inactivated and HIF-1α is transported into the nucleus where it forms a heterodimer with HIF-1β, binds with co-activators and activates expression of a vast array of genes having one common denominator: the presence of the hypoxia responsive element (HRE) 5'G/ACGTG3' in their promoter sequence.

The HRE element, located upstream of transcription start site of the *CA9* promoter is the main *cis*-acting regulator of CA IX expression [28]. Furthermore, five additional regulatory DNA sequences, PR1-5 ('Protected regions', denominated after regions identified in DNaseI footprinting assays) were identified (Fig. 11.1) [35]. PR1-3 and PR5 were proven to regulate CA IX expression in a positive

manner [35, 36]. These *cis*-acting elements are recognized by following *trans*-acting factors: PR1 and PR5 regions interact with SP1/SP3 (Specificity protein1 and 3) transcription factors; PR2 is bound by AP-1 (Activator protein-1) transcription factor and PR3 interacts with nuclear extract proteins [31, 35–37]. According to promoter deletion analysis, PR1 and PR2 are essential for CA IX transcription [35]. In contrast, PR4 is a negative regulatory element acting as a silencer of CA IX expression [35]. The region is bound by a repressor complex comprising MORC2 (Microrchidia 2) and HDAC4 (Histone deacetylase 4) proteins [38]. The proposed mechanism suggests that MORC2 recognizes the PR4 element and HDAC4 in turn catalyzes deacetlyation of histone H3 in the *CA9* promoter region and thus perturbs its transcriptional activation [38].

It is noteworthy that CA IX transcription can be also initiated in normoxia, by high density of in vitro cultivated cells. Higher cell density causes a decrease of pericellular oxygen levels defined by less than 5 % and more than 1 % of oxygen; a condition referred to as 'mild hypoxia' [36]. Such levels of oxygen are sufficient to trigger proteasome degradation of HIF-1α but nevertheless, CA IX is expressed [39]. This phenomenon stimulated the search for signaling pathways that may potentially influence CA IX expression in these conditions.

In mild hypoxia, CA IX expression is induced by PI3K pathway mechanism [39]. To trigger CA IX expression, only the HRE element and PR1 region are required; however, the process is still dependent on minimal HIF-1α activity [39]. This conclusion supports the ultimate dependence of CA IX expression on HIF-1 activation. In addition, high cell density-induced mild hypoxia has additive effect on true hypoxic conditions, considerably increasing the CA IX expression levels [39]. Furthermore, the expression of CA IX is regulated by components of MAPK pathway both in true hypoxic and mild hypoxic conditions [40]. Simultaneous inhibition of components of the PI3K and MAPK signaling pathways leads to a dramatic drop of CA IX transcription activity [40].

5.2 Regulation of CA IX Activity

The capacity of CA IX to acidify the extracellular environment is dependent on specific post-translational modifications within its IC tail.

The cytosol-protruding CA IX IC tail is involved in cell membrane targeting of assembled CA IX [41]. Deletion, as well as targeted mutagenesis experiments revealed the major role of CA IX IC tail in cell surface localization of the protein, along with the management of its pH regulating and ECD shedding capacity [41]. The CA IX IC tail contains several putative phosphorylation sites. Among these, Tyr^{449}, Thr^{443} and Ser^{448} bear the potential to regulate CA IX activity [42, 43]. Epidermal growth factor (EGF)-induced Tyr^{449} phosphorylation mediates cross-talk between CA IX and PI3K and in this manner contributes to Akt kinase activation [42]. However, the impact on CA IX activity is yet undetermined [43].

In vitro substitution of Thr^{443} by a non-phosphorylatable amino acid perturbs the extracellular acidification capacity of CA IX [43]. On the contrary, phosphorylation of Ser^{448} affects CA IX activity in a negative manner. Subsequent experiments revealed that Thr^{443} residue is recognized and phosphorylated by Protein kinase A (PKA) in vivo [43]. The relation of CA IX activity and PKA phosphorylation illustrates another aspect of the definitive dependency of CA IX on hypoxia, since: (i) elevated intracellular concentration of cAMP required for PKA activity; and (ii) elevated levels of active PKA (Catalytic subunit of PKA phosphorylated on Thr^{197} residue) were observed in hypoxia compared to the normoxic conditions [43].

As for the phosphorylation status of Ser^{448} as a negative regulator, the in vivo evidence is at the moment missing, and so is the putative kinase involved in the process, therefore further experimentation will be required in this matter [43].

5.3 Regulation of CA IX Abundance

Total cell surface population of CA IX is regulated by metalloproteinase-mediated cleavage of CA IX ECD in a process termed 'ectodomain shedding' [44, 45]. The CA IX ECD comprises CA and PG domains and is represented by a 50/54 kDa twin band on Western blots [44]. The basal rate of 'shed' ECD represents up to 1/5 of the membrane-bound CA IX population [45]. Experimental data revealed that CA IX ECD shedding is induced by TNFα-converting enzyme (TACE/ADAM17) [45]. However, the biological impact of ECD shedding still awaits its identification.

6 Role of CA IX in Cancer

6.1 CA IX Expression in Normal and Cancer Tissues

CA IX is limitedly distributed among normal tissues with almost a total exclusivity in the gastrointestinal tract epithelium, namely glandular gastric mucosa, epithelium of the gallbladder and cryptic enterocytes of duodenum, jejunum and ileum [3, 46, 47]. Also, the expression in ovarian coelomic epithelium, pancreatic ductal cells, cells of the hair follicles, and fetal rete testis has been observed [48, 49]. In contrast, the protein is ectopically expressed in a variety of cancer tissues. The expression of CA IX was observed in many tumors including malignancies of the brain, head/neck, breast, lung, bladder, cervix uteri, colon/rectum and kidney [48, 49]. cDNA comparison of CA IX sequence obtained from normal and tumor tissues showed no difference between these sequences assuming regulatory intervention into CA IX expression [47]. Indeed, the overexpression of CA IX in these solid tumors is driven by hypoxia, with the exception of clear cell renal cell carcinoma (CCRCC), where the overexpression is triggered by a different mechanism.

As mentioned earlier in this chapter, in normoxia, the level of HIF-1α transcription factor is regulated by pVHL-mediated polyubiquitylation and subsequent degradation in the proteasome. However, in CCRCC, CA IX expression throughout the tumor tissue was detected even in normoxia [50]. The responsible mechanism was revealed after the two seemingly independent lines of research were brought together.

6.2 CA IX as a Diagnostic Marker

In 1986, using monoclonal antibody G250, a novel marker specific for renal cell carcinoma (RCC) was discovered [51]. The marker showed homogenous expression at a high level throughout the tumor, in contrast to almost no expression in normal tissues despite a few exceptions. Interestingly, the marker was discovered in other types of tumors as well, although the expression was rather heterogenous and at a lower rate [52]. The identity had not been elucidated until 2000, when G250 cDNA was analyzed, revealing 100 % identity with CA IX [13, 52]. By a fusion of the two lines of research, it became evident that hypoxia and namely HIF-1α are responsible for the heterogeneous expression in many types of solid tumors, whereas inactivating mutations of the VHL tumor-suppressor determine the normoxic expression in RCC [52].

Approximately 60–80 % of all RCC patients carry inactivating mutations of the VHL tumor-suppressor gene in their genome [53]. In this manner, HIF-1α subunit is stabilized even in normoxia and constitutive CA IX expression is administered. Among different RCC subtypes, CCRCC is associated with the highest CA IX expression. The protein is expressed in 97 % of CCRCC cases with apparently no expression in normal renal tissue [48]. Therefore, CA IX bears the potential of being a diagnostic marker for renal malignancies. However, its expression is not only connected with numerous RCC subtypes, yet with a whole spectrum of solid tumors. Large-scale studies of tumor-derived cell line and tissue specimens revealed CA IX expression in specific carcinomas of the cervix uteri and uterine corpus, ovary, gastrointestinal tract, liver, pancreas, lung, head/neck, salivary gland, body cavity and skin [48, 49]. CA IX is thus rather a general marker of tumor hypoxia in many solid tumors, than a specific marker for distinct types/subtypes of malignancies.

6.3 CA IX as a Prognostic Marker

The importance of detecting CA IX ectopic expression in human tumors lies not only in its diagnostic nature. Interestingly, the level of CA IX expression is also associated with staging and survival prognosis in several types of human tumors. Higher levels of CA IX expression are associated with poor clinical outcome in cervical, rectal, breast, lung, and brain tumors [54–58]. In contrary, *low* expression

levels of CA IX seem to indicate poor prognosis in CCRCC [59]. However, this notion has been opposed by a larger-scale study suggesting that there is no relevant connection between low CA IX expression and poorer prognosis in CCRCC [48]. The discrepancy might be related to a very high cut-off value (of 85 % CA IX-positive cells in a tissue), which was proposed to discriminate between high and low expression of CA IX [59].

Generally, the very impact of CA IX pH regulating activity on tumor behavior might be connected with poorer outcome, rendering the protein a promising biomarker and drug therapy target.

6.4 CA IX and pH Regulation in Solid Tumors

The events leading to formation of two hallmarks of solid tumors, *hypoxia* and relative *tumor acidosis* have been described earlier in this chapter. Briefly, rapid advancement of tumor tissue can be characterized by several phenomena summing up to the final attributes of the tumor microenvironment. Uncontrolled growth and proliferation lead to rapid increase of the tumor tissue. As the rate of angiogenesis ceases to match its demand, areas with true hypoxia develop in parts of the tumor most distal from the functional blood vessels. To address the reduced oxygen supply, HIF-1 induces the expression of HRE element-carrying target genes that organize the reconstruction of cellular behavior under true hypoxic conditions. The cancer cells shift their metabolism to anaerobic glycolysis to maintain energy production required for continuous proliferation. One of the key characteristics of glycolytic metabolism in hypoxic conditions is increased production of acidic metabolites that are poorly removed from extracellular space due to insufficient vasculature.

To summarize, hypoxia, and namely its right hand, tumor acidosis, represent toxic environmental conditions to which normal cells do not possess the means of adapting. Hence, the ability to regulate pH_i is the key selective advantage enabling to overcome the life-limiting stress represented by acidic tumor microenvironment.

7 Introduction to Tumor Acidosis

It is noteworthy that low pH in the tumor microenvironment supports the growth of cancer cells by several independent lines of impact. Acidic milieu is unfavorable for normal cells, as well as for immune cells that could recognize tumor antigens [23]. Decrease of pH_i leads to several major consequences ranging from disruption of ATP synthesis and alternative splicing of various proteins to p53-mediated apoptosis by the activation of caspases [5, 60]. It has also been discussed that acidic pH_e increases invasive behavior of cancer cells thus enhancing their metastatic potential [61]. In addition, low pH_e induces angiogenesis by upregulation of vascular endothelial growth factor (VEGF) expression in cancer cells [62].

Principally, excessive concentrations of lactic acid emerging from the glycolytic cycle were believed to acidify pH_e [63, 64]. However, experiments on lactate dehydrogenase (LDH) deficient cells proved that carbon dioxide may represent equally potent cause of pH_e decrease [64]. Either way, acidic pH_e is the direct result of the activity of pH_i regulators in cancer cells.

8 CA IX and pH Regulation in Cancer Cells

Simplified, two main processes represent the pH_i regulating mechanisms of tumor cells: pH_i buffering and acid extrusion. To position the place of CA IX within the complex pH regulating machinery in cancer cells, its enzymatic reaction needs to be highlighted. As mentioned earlier in this chapter, CA IX reversibly catalyzes carbon dioxide hydration to bicarbonate ions and protons by the reaction that is taking place in the extracellular space. To maintain acid extrusion from cells, carbon dioxide needs to be hydrated in the extracellular space and not in the cytosol [65]. In order to achieve this, the hydration of carbon dioxide needs to be catalyzed by CA IX which effectively facilitates the reaction so that the carbon dioxide concentration gradient is directed extracellularly [65]. Bicarbonate ions are transported into the cell by Na^+/HCO_3^- cotransporter (NBC), Na^+-dependent Cl^-/HCO_3^- (NDCBE) exchanger and by members of the anion exchanger (AE) family [24]. Interestingly, several lines of evidence indicate that CA IX may directly interact with the AE2 and NBC according to a metabolon concept, thus representing even more interconnected and adaptive pH regulating system (Fig. 11.2) [6, 66, 67]. This,

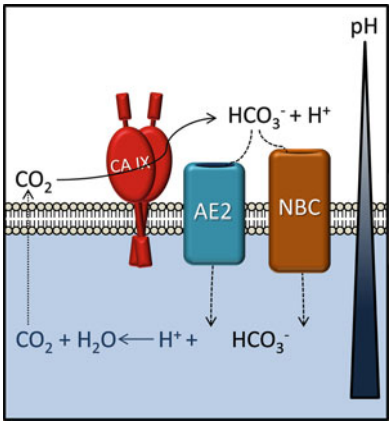

Fig. 11.2 The metabolon concept. CA IX catalyzes the hydration of carbon dioxide to bicarbonate ions and protons. By direct interaction with Na^+/HCO_3^- cotransporter (NBC) and Anion exchanger 2 (AE2) transporters, bicarbonate ions are immediately transported into the cell, where they interact with protons liberated by glycolysis. The reaction represents the basis of intracellular pH alkalinization. Carbon dioxide, the final product, then diffuses through the plasma membrane where it contributes to extracellular pH acidification

along with the fact that downregulation of individual proton transporters potentially perturbs tumor growth, demonstrates the complexity and extensive dynamics of pH balance maintenance in cancer cells [24].

The pH_i buffering activity of cancer cells is reinforced by acid extrusion. In line with CA IX-mediated carbon dioxide hydration, several additional acid extrusion mechanisms contribute to pH_i alkalinization and simultaneous extracellular pH acidification. One of the most important pH regulators functioning by H^+ extrusion is NHE isoform 1 (NHE-1) [65, 68]. Its crucial role in tumorigenesis was demonstrated in NHE-1 activity deficient cells that showed significantly decreased rates of tumor growth [24]. Proton extrusion is also maintained by vacuolar-type (V-type) H^+ ATPases [69]. The expression of V-type ATPases furthermore confers anti-apoptotic attributes to the cancer cells [65]. Finally, monocarboxylate transporters (MCTs) play an essential role in acid extrusion by exporting pyruvate and lactate along with protons, thereby being inevitable for pH_i alkalinization of cancer cells [70].

As mentioned previously, consequently to the above-mentioned processes the pH of the extracellular milieu is decreasing. In addition to poor removal of extruded acidic metabolites and protons, the activity of CA IX was proven to significantly reduce extracellular pH in hypoxia [20]. Experiments on immortalized CA IX-negative MDCK (Madin-Darby canine kidney) cell line compared to MDCK cells constitutively expressing CA IX after stable transfection revealed a major impact of CA IX CA domain on pH_e decrease. The concept was validated by experiments using selective CA IX inhibitors on HeLa (cervical cancer) and SiHa (cervical cancer) cancer cell lines and ΔCA CA IX isoform in HeLa cells [20].

Excessive acidification of pH_e due to the activity of cellular acid extrusion mechanisms and the activity of CA IX are not only connected with cancer cell survival in conditions that are unfavorable for normal cells. By several lines of action, tumor acidosis stimulates the metastatic potential of cancer cells.

8.1 The CA IX Involvement in the Metastatic Cascade

Accumulation of genetic disorders that led to the creation of cancer cell genotype may not alone define the aggressive phenotype of most cancers, as the possibilities of primary tumor growth are limited [5]. Invasion to adjacent tissues and distant organs, i.e. the metastatic process represents one of the conditions defining poor curability of patients [71]. Metastatic process is gradual and comprises several steps including reduction of cell-cell adhesion, degradation of extracellular matrix (ECM) components, invasion and migration into surrounding tissues or lymphatic and blood vessels, re-adhesion and finally proliferation and angiogenesis [71, 72].

Tumor acidosis, fuelled by the activity of pH_i regulating mechanisms of cancer cells stimulates their potential to metastasize. The involvement of low pH_e in the metastatic cascade is supposed to be both indirect and direct; by p53-mediated apoptosis of normal cells surrounding the tumor tissue and by promoting

basement membrane degradation in acidic regions of the tumor, respectively [5, 61]. The degradation of the basement membrane represents the onset of cancer cell dissemination into distant organs and tissues, impersonating the barrier between metastatic and non-metastatic cancers. By overcoming this barrier, cancer cells are free to proliferate into the surrounding tissues. Acidic tumor microenvironment stimulates secretion of cathepsin B, a protein which plays a significant role in degradation of the components of the ECM including the basement membrane [73]. In addition, members of the matrix-metalloproteinase (MMP) family and the urokinase-like plasminogen activator (uPa) are believed to be secreted in acidic tumors [73, 74]. Finally, acidic pH_e alters the size, abundance and intracellular distribution of lysosomes thereby enhancing the secretion of ECM degrading proteinases [61, 74, 75].

It is noteworthy that CA IX also actively participates in the metastatic process. The protein is connected with the reduction of cell-cell adhesion and the migratory phenotype of cancer cells.

8.1.1 CA IX and Cell Migration

Cell adhesion is mediated by adhesion molecules, including cadherins, selectins, integrins and immunoglobulin protein family members. Of these, E-cadherin is in the spotlight because its level of expression often correlates with prognosis of the malignancy [76]. E-cadherin is a 120 kDa transmembrane protein expressed on the basolateral surface of epithelial cells [76, 77]. Its extracellular domain plays a key role in cell-cell adhesion by homotypic interaction with another E-cadherin molecule protruding from adjacent cell. The cytoplasmic domain of E-cadherin interacts with proteins of the catenin family, namely with β-catenin [78]. β-catenin is recognized and bound by α-catenin which in turn mediates the connection with actin cytoskeleton [79]. Furthermore, the E-cadherin-catenin complex is modified by WNT-1 protein, which recognizes β-catenin [76]. The catenin-mediated connection between E-cadherin and actin cytoskeleton is the essence of cell-cell adhesion.

Disruption of cell-cell adhesion is the initiating step of the metastatic cascade. In order to detach from the primary tumor mass, cancer cells undergo a series of morphological and behavioral changes, most prominent of which is the epithelial-mesenchymal transition (EMT). By EMT the morphology of polarized epithelial cells is rebuilt to that of migration prone mesenchymal cells by the activity of various transcription factors including Slug, Twist, Snail or by environmental conditions such as hypoxia [72, 80]. The morphological changes include cytoskeletal reorganization; polarization of the cancer cell along the axis of the movement and the formation of lamellipodia and invadopodia [81]. Formation of the leading edge of the migrating cell is followed by its detachment from the matrix and the formation of a trailing edge [82].

In the process of cell migration, intracellular as well as extracellular pH plays a major role. Thus, tumor acidosis caused by the activity of pH_i regulating mechanisms of cancer cells directly influences their migration capabilities. According

to several studies, NHE-1 transporter has the most prominent role in affecting cancer cell migration [82–84]. Before migration, the re-localization of pH regulators including NHE-1, NBC, AE2, MCT and finally CA IX from their original position into the leading edge takes place [81]. Their activity generates pericellular as well as intracellular pH gradients. The pericellular pH gradient is directed from the leading edge to the trailing edge, while being the most acidic at the leading edge region. In contrary, due to the extensive activity of pHi regulating mechanisms at the leading edge, the intracellular pH gradient is rising from the trailing edge to the leading edge. The formation of pH_i gradient is one of the prerequisites of cell migration [82]. Interestingly, increased levels of cAMP were detected in lamellipodia and PKA was observed in the leading edge of migrating cells [85]. This, hand-in-hand with the metabolon concept, indicates that a complex network of closely related events takes place before migration is initiated; a network in which CA IX is one of the key players.

Indeed, the effect of CA IX on the promotion of cancer cell migration goes beyond its contribution to pH_i regulation. As observed in polarized MDCK cells, CA IX responds to calcium depletion by internalization along with E-cadherin, β-catenin and other adhesion molecules [86]. This behavior, typical for adhesion molecules was also observed in hypoxia. Upon further investigation of this phenomenon, it was revealed that CA IX interacts with β-catenin and thus interferes with the E-cadherin attachment to the actin cytoskeleton, hence, it perturbs E-cadherin-mediated adherent junctions between epithelial cells [86]. In other words, this competitive interaction destabilizes cell-cell adhesions and thus significantly supports cell migration. Experimentally, it was observed that ectopically expressed CA IX significantly increases migration rate of MDCK and HeLa cells in vitro, whereas ΔCA CA IX isoform lacks this ability [67]. Obviously, the catalytic activity of CA IX is required for this process. Furthermore, CA IX is presumably involved in activation of PI3K/Akt signaling pathway and the c-Src/FAK axis, and cross-talks with the Rho/ROCK signaling pathway, all of which are believed to increase the migration rate [42, 87, 88]. However, this function of CA IX might be regulated by DKK-1 protein interaction with the PG domain of CA IX, positioning the DKK-1 protein into the role of anti-tumorigenic agent [87].

Overall, the catalytic activity of CA IX, along with its role as cross-talk-mediator with essential migration-regulating pathways confirm not only its major contribution to cancer cell survival in conditions of environmental acidosis; the above mentioned functions of CA IX underline its direct involvement with aggressive and invasive phenotype of cancer cells.

8.2 CA IX as a Therapeutic Target

The above-mentioned characteristics of CA IX with respect to its significant role in tumorigenesis highlight the immense potential of CA IX-targeted cancer therapy. The discovery of its tumorigenesis-related activities has led to extensive search for

selective CA IX inhibitors. On the level of all CAs, acetazolamide is one of the best studied inhibitors of CA activity, with proven contribution to the reduction of invasiveness of renal cell carcinoma-derived cell lines [89]. For more specific targeting, the drug-design strategy was focused on compounds bearing positive or negative charge, thus being unable to enter the cell [90]. Hence, the compounds can interact only with extracellular CA isoforms. Furthermore, mass spectrometric and X-ray crystallographic analyses of CA IX structure opened the door for site-specific drug design [7, 17, 18]. Determining that the active site (region 125–137, in particular) of the CAs is the most variable, these studies enabled to direct the activity of CA inhibitors against specific CA isoforms [17].

Throughout the years, many CA IX-selective compounds with various mechanisms of action were identified, most prominent of which are sulfonamides, their isoesthers (sulfamates, sulfamides) and coumarins [90]. Sulfonamides, both aromatic and heterocyclic, e.g. homosulfonilamide and 1,3,4-thiadizole-2-sulfonamide, respectively, were proven to inhibit specifically CA IX activity [22]. The mechanism of sulfonamide inhibiting action is based on binding of the sulfonamide anion to the zinc ion inside the enzyme active site [91, 92]. In contrast, the mechanism of coumarin-mediated CA IX inhibition is based on spatial blocking of the CA IX active site, upon binding of the hydrolyzed form of coumarin to its entrance [90, 91].

A different approach to target exclusively CA IX is the utilization of CA IX-specific antibodies. Monoclonal anti-CA IX antibodies have been successfully utilized for CA IX detection and visualization of hypoxic areas in solid tumors. Among the numerous CA IX specific antibodies used for this purpose, it is important to mention M75, G250 and its chimeric derivative (cG250) conjugated with radionuclides, and high–affinity A3 and CC7 human monoclonal antibodies [51, 93, 94]. A step towards anti-CA IX immunotherapy was the introduction of mouse monoclonal antibody VII/20, which possesses internalizing capabilities [95]. The antibody may serve as a carrier for targeted anti-cancer therapy. Furthermore, using phage display technology, novel CA IX-specific antibodies with internalizing and inhibitory effect on CA IX activity were developed [96, 97]. Finally, RENCAREX® (Wilex AG), a cG250 antibody underwent phase I-III clinical trials for treatment of patients with RCC. Phase I-II showed promising effects on metastatic renal cell carcinoma patients' survival rate and tolerance of the antibody [98]. Although the recently finished phase III clinical trial (ARISER), targeted at patients with non-metastatic renal cell carcinoma showed no significant improvement of disease-free survival among patients treated with RENCAREX® compared to placebo, more careful evaluation of data revealed clinically and statistically significant improvement in disease-free survival in the patient population with a high CA IX level treated with RENCAREX® compared to both placebo and patients with a low CA IX score (see http://www.wilex.de/press-investors/announcements/press-releases/20130226-2/). This is in agreement with earlier investigations, which showed that the efficient targeting of tumor cells is affected by antigen heterogeneity and requires certain CA IX antigen density [99].

9 Conclusion and Future Perspectives

After almost two decades of extensive research, the role of carbonic anhydrase IX in cancer has been found to be far more complex than originally imagined. The wealth of experimental data demonstrates the ultimate dependency of CA IX expression, assembly and activity on the hypoxic microenvironment. It is remarkable that its direct connection with tumor hypoxia and microenvironmental acidosis was experimentally proven to contribute to development of stress conditions that are toxic for normal cells but not for adaptable cancer cells. The involvement of CA IX in cell-cell adhesion, cell migration as a part of the metastatic cascade, as well as the observed increase of the invasiveness of cancer cells designate its strong position in the process of tumorigenesis. In this manner, CA IX represents an ideal target for anti-tumor therapy. The potential of CA IX-targeted anti-tumor therapy is even broadened by its rare expression in normal tissues and cell-surface localization. The advancements in the design of CA IX inhibitors, which enable to target CA IX only in hypoxic conditions, along with the prospect of CA IX-mediated immunotherapy represent a challenge for the following research. Both approaches follow perspective principles that are promising for translation into the clinic. At the same time, the knowledge of CA IX activators, putative interacting partners, as well as its potential role in other tumor-related phenomena acquired *in silico* and in vitro might bring interesting insights into new horizons of CA IX-targeted therapy in the near future.

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