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A study of Physiological Homeostasis and its Influence of Tumor pH on Therapeutic Response

Dr. Rakesh Kumar Godi
Professor, Department of information Science and Engineering, PDACEG

Abstract *: The intratumor microenvironment is intrinsically acidic due mainly to accumulation of lactic acid as a result of increased aerobic and anaerobic glycolysis by the tumor cells. In general, the extracellular pH (pHe) in human tumors is below 7.0, whereas the intracellular pH (pHi) is maintained at neutral range, i.e., >7.0, by powerful pHi control mechanisms. The low pHe and the significant gradients between pHe and pHi affect markedly the response of tumors to various treatments such as chemotherapy, radio-therapy and hyperthermia. For instance, the acidic pHe increases the cellular uptake of weakly acidic drugs such as cyclophosphamide and cisplatin and thus increases the effect of the drugs, whereas the acidic pHe retards the uptake of weakly basic drug such as doxorubicin and vinblastine, thereby reducing the effect of the drugs. The radiation-induced apoptosis is suppressed by an acidic environment, whereas the hyperthermia-induced cell death is potentiated by an acidic environment. Better understanding of the control mechanisms of pHe and pHi in tumors may lead to device effective treatment strategy of human tumors.****Key Words:*** *Intratumor pH; extracellular pH; intracellular pH; intratumor pH con-trol mechanism; chemotherapy; radiotherapy; hyperthermia.*

1. Introduction

The environmental acidity or pH of living cells and tissues is one of the major factors that influence molecular processes involved in cell cycle progression, cell proliferation, and differentiation. Likewise, oncogenesis, malignant transformation, metastasis, and angiogenesis are greatly influenced by environmental acidity. The environmental acidity also greatly influences the response of cancer cells to various treatments. The vascular network in tumors is inhomogeneous, causing insufficient oxygen supply to parts of tumors. The resultant hypoxia forces glucose metabolism through the glycolytic pathway

instead of respiration, thereby resulting in the formation of lactic acid *(1–7)*. In addition, tumor cells convert glucose and other substrates preferentially to lactic acid and other acidic metabolites even under aerobic conditions, leading to acidification of the intratumor environment *(5,8,9)*. Whereas the interstitial or extracellular environment in tumors is acidic, the intracellular pH (pHi) in tumors has been found to be at neutral range, i.e.,< 7.0, similar to the pHi of normal tissues *(1,2,5–12)*. This intracellular and extracellular pH (pHe) gradient in tumor cells is maintained by sophisticated biophysical mechanisms *(1,2,13)*. It has been demonstrated that the gradient between pHe and pHi of tumor cells renders the cells resistant to weakly basic drugs by hindering the cellular uptake of the drugs, whereas the same pH gradient increases the uptake of weakly acidic drugs. The influence of tumor acidity on the thermosensitivity of tumor cells has been extensively investi-gated. On the other hand, relatively little has been revealed on the effect of acidic intratumor environment on the response of tumor cells to radiotherapy. In this chapter, we review the pHi control mechanisms and the implications of tumor pH and that of the pH gradient between the outside and inside of tumor cells on the response of tumor cells to various treatments.

2. TUMOR pH

It has long been known that the microenvironment in tumors of both animal and human is acidic as compared with that in normal tissues because of elevated in anaerobic as well as aerobic glycolysis in tumors *(1–6)* . As tumor nodules are formed, neovascularization begins from host venules stimulated by a number of angiogenic factors secreted by the tumor cells as well as adjacent normal cells. The newly formed tumor vascular beds are characterized by a heterogeneous distribution of dilated, irregularly bulged, constricted, twisted, and sharply bent capillary-like blood vessels *(14–23)*. Consequently, tumor blood perfusion is sluggish, resulting insufficient supply of various nutrients, including oxygen, to tumor cells. As the tumor grows larger, the intercapillary distance progres-sively increases, and areas beyond oxygen diffusion distance from capillaries, i.e., about 150 m, become hypoxic *(24)*. In addition, probably because of progressively increasing interstitial pressure caused by the increasing tumor cell population *(25)*, tumor blood vessels are compressed, and the blood perfusion ceases intermittently or permanently resulting in intermittent or permanent hypoxia *(20–23)*. Hypoxia upregulates various transcription factors including hypoxia -inducible factor -1 (HIF-1), which activates the transcription of numerous genes whose protein products facilitate adaptation to hypoxia, driving the tumor toward a more malignant phenotype *(26–28)*. A well-known response of cells to hypoxia is an increase in hyperglycolytic activity characterized by increased

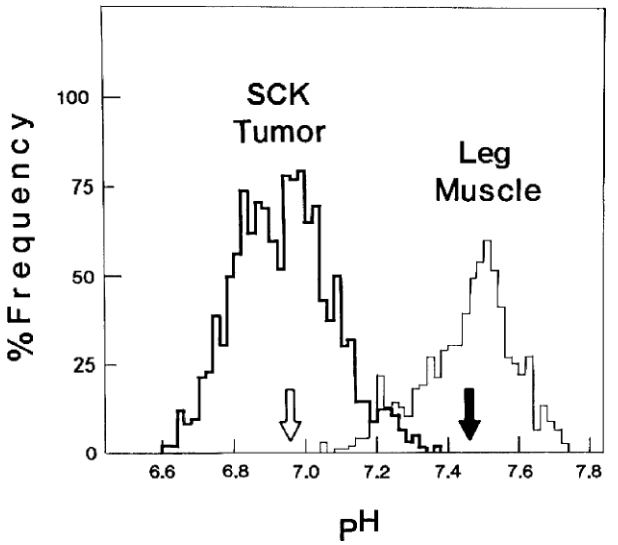

Fig. 1. Histograms of interstitial pH in the leg muscle of A/J mice and that in SCK tumors grown subcutaneously in the leg of A/J mice. The tumor diameters were 7–9 mm. The pH was measured with glass microelectrodes 50–80 mm in diameter *(3)*.

glucose uptake and formation of lactic acid, resulting in acidification of intratumor en-vironment *(5–7)*. It has also been demonstrated that hypoxia activates carbonic anhy-drase, thereby causing hydration of CO_2 molecules to carbonic acid *(7)*. Hulikova et al. *(29)* reported that tumor -associated carbonic anhydrase IX isoform is the most likely candidate involved in the formation of carbonic acid under hypoxic conditions. Hydroly-sis of ATP is also a significant

contributor to acidosis in tumors during acute hypoxia *(30)*. It should be pointed out, however, that tumor acidification can occur independent of hypoxia. It was shown in the early part of the last century that tumor cells metabolize glucose preferentially through glycolysis, even in the presence of oxygen *(8)*. It is believed that the endogenous acidification is an integral property of tumor cells that may have evolved to provide tumor cells with a competitive advantage over stromal cells *(5,8)*. Elstrom et al. *(31)* reported that the high rate of aerobic glycolysis in cancer cells is because of upregulation of the serine/threonine kinase Akt *(9)*.

2.1. pH in Tumors vs Normal Tissues

Until recent years, pH of animal and human tumors was determined by glass or fiber optic pH electrodes *(3,4,32–35)*. Because the diameters of the electrodes are larger than the diameters of cells and the tissue damage caused by the electrodes can be substantial, the pH values obtained with microelectrodes represent mainly pHe. Despite the technical difficulties, important information on tumor pH has been accumulated during the last several decades. Figure 1 shows histograms of pHe in SCK mammary carcinoma and that of the leg muscle of A/J mice obtained with glass microelectrodes *(3)*. It is demonstrated that the pHe in SCK tumors ranged from 6.60 to 7.38, with a mean value of 6.96, whereas that of the muscle ranged from 7.05 to 7.72, with a mean value of 7.45. This difference between mean tumor pH and mean muscle pH of as much as 0.5 pH units means that the concentration of the active H^+ ions in the interstitial space in SCK tumors was five times

greater than that in the muscle. Wike-Hooley et al. *(4)* reviewed a number of reports on the pHe value in tumors and normal tissues of animals and concluded that the tumor pHe ranged from 5.8 to 7.68, with an average of 7.09, and that the pHe in normal tissues such as muscle and liver was about 0.5 pH units higher than that in tumors. Vaupel et al. *(36)* reported that whereas average pHe in a C3H murine mammary carcinoma was 6.7, the pHe in some microareas was as low as 5.8–6.3. On the other hand, the pHe measured in extensively necrotic areas was higher than that in normal tissues, probably because of lack of formation of acidic metabolites as a consequence of previous cell death. Jahde et al. *(37)* observed that the pHe in neuroectodermal TV1A tumors grown subcutaneously in the flank of BDIX rats ranged from 6.8 to 7.1, with a mean of 7.0. Interestingly, the pHe values in the brain and kidney of BDIX rats were similar to that measured in brain tumors of the same animal.

Meyer et al. *(38)* reported as early as 1948 that the pHe of human tumors was lower than that in normal tissues, and other investigators subsequently reported similar results *(4,32,39–49)*. Wike-Hooley et al. *(4)* also reviewed the distribution of pHe in human tumors and normal tissues. The tumor pHe ranged 6.0–7.6, with a median pHe of 7.1, whereas the subcutis/muscle pHe ranged 7.3–7.8, with a median pHe of 7.55. It has been reported that, in general, the range of pH values in tumors is much greater than that the normal tissues, probably because the distribution of the vascular supply and blood per-fusion in tumors are heterogeneous *(3,4)*. In this regard, the intertumor pHe variance was more striking than the intratumor pHe variance *(4)*. Based on numerous reports, Wike-Hooley et al. *(4)* concluded that the pH values in human tumors were not related to the tumor histology, degree of differentiation, tumor size, patient age, or treatment histology. However, the pHe values in metastases were higher than those in the primary tumors of a given patent.

2.2. Intracellular pH

It has become increasingly evident in recent years that pHi is not equal to pHe in tumor cells. We have studied the pHi of tumor cells in vitro using the pH-sensitive dye BCECF *(1,2)*, as shown in Fig. 2. The pHi remained at about 7.4 when the medium pH, i.e., pHe, was in the 7.0–7.4 range. As the pHe was lowered, the pHi also decreased, but only slightly. For example, at pHe 7.0 and 6.0, the pHi was 7.4 and 6.7, respectively. This in vitro study demonstrated clearly that pHi of tumor cells in a low-pHe environment re-mains near the neutral pH range. It has become possible in recent years to determine pHi of tumor cells *in situ* by virtue of impressive progress in magnetic resonance spectroscopy (MRS) technology. The pHi of tumors has been measured with ^{31}P-nuclear magnetic resonance (NMR), which determines the shifts in intracellular inorganic phosphate and phosphocreatine *(10,50–54)*. It is now possible to determine pHe using ^{1}H-MRS and also simultaneously determine pHi and pHe by incorporating a pHe indicator, 3-aminopropyl phosphonate, into ^{31}P-NMR *(6)*.

Fig. 2. Relationship between extracellular pH (pHe) and intracellular pH (pHi) of SCK tumor cells in vitro. The cells were maintained at pHe 7.2 before exposure to a new pHe. The pHi was measured using the pH-sensitive dye BCECF method 20–30 min after exposure to new pHe.

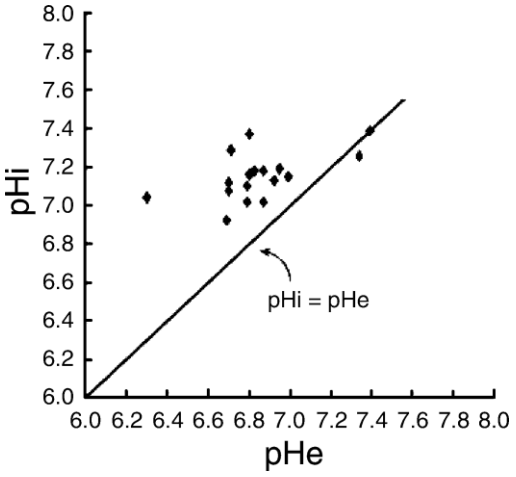

Fig. 3. The relationship between extracellular pH (pHe) and intracellular pH (pHi) in human, rat and mouse tumors *in situ*. Both pHe and pHi in the same tumors were determined with magnetic resonance imaging/magnetic resonance spectroscopy method. Data reported by ref. *6* were used to construct this figure.

and rat tumors determined with MRS/magnetic resonance imaging method and reviewed by Gilles *(6)* . It can be seen that the pHi values are higher than the pHe values in the same tumors in all tumors studied. The pHe values were correlated with phenotype, and the pHe values in larger tumors were lower than that in smaller tumors, probably because of poorer blood perfusion in larger tumors and more accumulation of acidic byproducts of glycolysis *(6)*. In conclusion, all available evidence indicates that the intracellular envi-ronment in tumor cells is less acidic as compared with extracellular environment in vitro as well as in vivo.

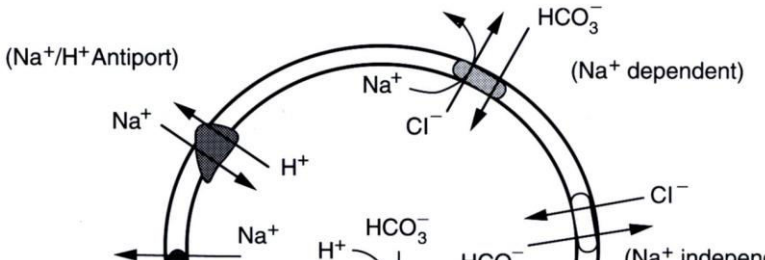

Fig. 4. Most common membrane-based intracellular pH regulatory mechanisms in mammalian cells.

3. Mechanism of pH_I Control

The fact that pHi is significantly higher than pHe in tumors demonstrates the existence of powerful mechanisms to prevent acidification of the intracellular environment *(13,55– 61)*. Such significant gradient between pHe and pHi has been attributed to existence of short-term and long-term mechanisms for pHi control *(13)*. The short-term mechanisms are essentially rapid buffering responses against an acute acid load in the cytosol of cells. The most important short-term regulatory mechanism is the physiochemical buffering of the acids. Other rapid mechanisms include metabolic consumption of nonvolatile acids and transfer of acids from the cytosol to the organelles. These three mechanisms are only for rapid consumption of H^+ ions to minimize rapid acidification in the cells; therefore, their capacity to maintain the intracellular environment at neutral pH for a prolonged period is limited. Almost all mammalian cells that have been investigated thus far possess powerful systems to regulate pHi using several long-term mechanisms *(13)*. The most important mechanism for long-term pHi regulation is the exchange of Na^+ ions for H^+ ions using the Na^+/H^+ antiport, an ion exchanger in the plasma membrane (Fig. 4) *(56,57)*. This process is believed to occur by the binding of intracellular H^+ ions

to the cytoplasmic surface of the exchanger and the binding of Na^+ ions to the cell surface of the exchanger. However, indications are that the exchange of Na^+ ions and H^+ ions is not a simple one-for-one exchange. It has been postulated that there might be a second cytoplasmic H^+ binding site that allosterically activates the antiport *(56,59)*. The influx of Na^+ ions and efflux of H^+ ions by this antiport is driven by a Na^+ gradient across the cell membrane. However, even in the presence of large Na^+ gradient energy, the exudation of H^+ ions from the cells is limited, and the pHi is stabilized at neutral values. This fact indicates that although the Na^+ gradient is important for the Na^+/H^+ exchange, it is not the only factor that controls the pHi. The antiport may become inactive when the pHi reaches a certain level, even though the Na^+ gradient remains large *(56)* . When the extracellular Na^+ ion concentration is low, the Na^+ gradient is reversed, and H^+ ions will enter the cells *(56)*. The Na^+ ions that enter the cells are extruded from cells driven by ATP hydrolysis. The activity of the Na^+/H^+ antiport is partially reduced under hypoxic conditions, which may be attributed to the reduction of ATP content *(13,60)*. There is evidence that the Na^+/H^+ antiport system is secondarily dependent on the Na^+/K^+-ATPase *(60)*. A number of compounds have been demonstrated to interfere with the Na^+/H^+ antiport. Amiloride, a diuretic drug and weak base, and many of its analogs inhibit the Na^+/H^+ antiport activity by competing with Na^+ ions for the Na^+ channel *(9,11,13,57,58,61)*. Ethylisopropylami-loride, an analog of amiloride, is a more specific inhibitor of Na^+/H^+ antiport than amiloride, and as such, ethylisopropylamiloride is a much more potent inhibitor of Na^+/H^+ antiport than amiloride *(12,57)*.

The intracellular acidity is also regulated by bicarbonate-linked mechanisms, namely

(1) Na^+-dependent Cl^-/HCO_3^- exchange, (2) Na^+-independent Cl^-/HCO_3^- exchange, and

(3) Na^+/HCO_3^- symport (*see* Fig. 4) *(11,13,55,57,58)*. All three mechanisms are not always present in all types of cells. Usually, various combinations of the three

mechanisms are found in different cell types. Among these, Na^+-dependent Cl^-/HCO_3^- exchange is probably the most important bicarbonate-linked mechanism for pHi control in mamma-lian cells. It responds only to acid challenge and neutralizes the intracellular environment by exchanging the negatively charged intracellular Cl^- with the extracellular Na^+/HCO_3^- complex *(62,63)*. The exchange is believed to be driven by Na^+ gradient and in some circumstances, by an additional inward-directed HCO_3^- gradient. The Na^+- independent Cl^-/HCO_3^- exchange is involved in protecting the cells from relatively rare occurrences of cell alkalinization. In this case, HCO_3^- ions are extruded from the cells, and Cl^- ions are transported into the cells to prevent the pHi from rising to an abnormally high level *(62,63)*. The Na^+/HCO_3^- symport is electrogenic, unlike the other two mechanisms *(64,65)*. A sudden reduction of Na^+ and Cl^- ions activates this mechanism to transport these two ions. Whereas this mechanism may be important for specialized acid-secreting cells, its role in regulating pHi in other mammalian cells is uncertain. All of the bicarbonate-dependent transporting mechanisms are inhibited by 4,4'-diisothiocyanostilbene 2,2'-disulfonic acids (DIDS), and 4-acetamindo-4' - isothiocyantostilbene 2,2' - disulfonic acids *(13,57,66–68)*. Ethacarynic acid inhibits the Na^+-independent Cl^-/HCO_3^- antiporter with-out affecting the Na^+- independent one, and picrylsulfonic acid has the opposite effect *(69)*. The Na^+-coupled Cl^-/HCO_3^- exchange is also inhibited by depletion of ATP, and the Na^+-independent Cl^-/HCO_3^- exchange is inactivated by a low-pH environment *(62,70)*.

The relative importance in maintaining pHi at neutral range of the different mecha-nisms mentioned varies markedly in different cell types and under different conditions. The lactate $/H^+$ symport, which is inhibited by the bioflavionoids quercetin and others, is one of the most active exchange in the regulation of pHi in tumor cells *(71,72)*. However, under hypoxic conditions the lactate extrusion is reduced, and so this exchange has little effect on resting pHi in the hypoxic cells *(5)*. In the gastric glands, the Na^+/H^+ antiporter plays the dominant role, whereas in the neighboring oxyntic cells, the Cl^-/HCO_3^- exchange plays the dominant role for the pHi regulation *(57)*. Three types of ATP-driven H^+ pumps have also been identified *(57)*. One of these is an ATPase-linked H^+ pump found in some specialized epithelial cells. It has been reported that one of the mechanisms to maintain the cytosolic pH at physiological level is sequestration of cyto-solic protons into acidic cellular vesicles such as endoplasmic reticulum, endosomes, and lysosomes. Interestingly, the ATPase -linked H^+ pump has been identified in a

number of intracellular organelles, indicating that the ATPase-linked H^+ pump plays an important role in regulating pH in the vesicles and cytosol. The other two mechanisms are a H^+-translocating

	Table 1		
	Genes Activated by Low pH		
	AP-1	VEGF	
	NF B	bFGF	K
	p53	PDECGF	
	p21	IL-8	
	MTIIA	Cyclines	
	GRPs	HSPs	
	Bax	NQO1	

ATPase and a K^+ /H^+ exchange ATPase, which can be suppressed by nigericin. Further understanding of the pHi control mechanisms may enable us to control the response of cells to internal as well as external stresses including various cancer treatments.

4. Effect Of pH On Angiogenesis and Metastasis

It has been established that hypoxic environment upregulates a number of transcrip-tion factors such as HIF-1, nuclear factor B, and activator protein 1 *(73–76)*. κHIF-1 has been demonstrated to activate transcription of as many as 70 genes including glucose transporters and glycolytic enzymes, which may account for the increased anaerobic glycolysis and resultant acidification of tumors under a hypoxic environment *(26–28)*. Like hypoxia, acidosis also upregulates transcription factors and activates a number of genes *(77–79)*. We have observed that exposure of tumor cells to a low pH medium elevates significantly p53 expression and p21 expression *(77)* . When the low pH medium was replaced with neutral pH medium, the

expression of p53 and p21 promptly returned to normal level. Table 1 shows some of the genes or their products upregulated by an acidic environment. Note that many of the genes activated by acidosis are the same genes activated by hypoxia. For example, the angiogenic factors such as vascular endothelial growth factor, basic fibroblast growth factor, platelet-derived endothelial cell growth factor, and interleukin 8 are upregulated by both hypoxia and acidosis. In view of the fact that many hypoxic cells in tumors are in an acidic environment, how the hypoxia and acidosis interact in promoting the angiogenic process remains an important avenue to be elucidated.

The metastatic potential of tumors has been demonstrated to be related closely to the environmental acidity. The ability of murine tumor cells to form lung metastases after intravenous injection increased significantly when the cells were cultured in acidic medium before the injection *(79,80)*. Deliberate exposure of mice bearing tumors to cyclic low-oxygen breathing (12 cycles of 5% oxygen breathing for 10 min interspersed with 10 min of air breathing) every day doubled the incidence of lung metastases *(81)*. It appeared that acidosis in combination with hypoxia induced by the low-oxygen breath-ing enhanced the incidence of metastasis. However, acidification of murine tumors by daily administration of metaiodobenzylguanidine and/or glucose without lowering tu-mor pO_2 did not enhance the spontaneous metastasis potential of tumor cells in the same model *(82)*. In addition to angiogenesis, induction of genomic instability or epigenetic regulation of gene expression may be involved in the increase in metastasis in acidic and hypoxic environments *(83)*. It is likely that the cells that survive the acidic and hypoxic hostile intratumor environment are more aggressive and metastatic as compared with

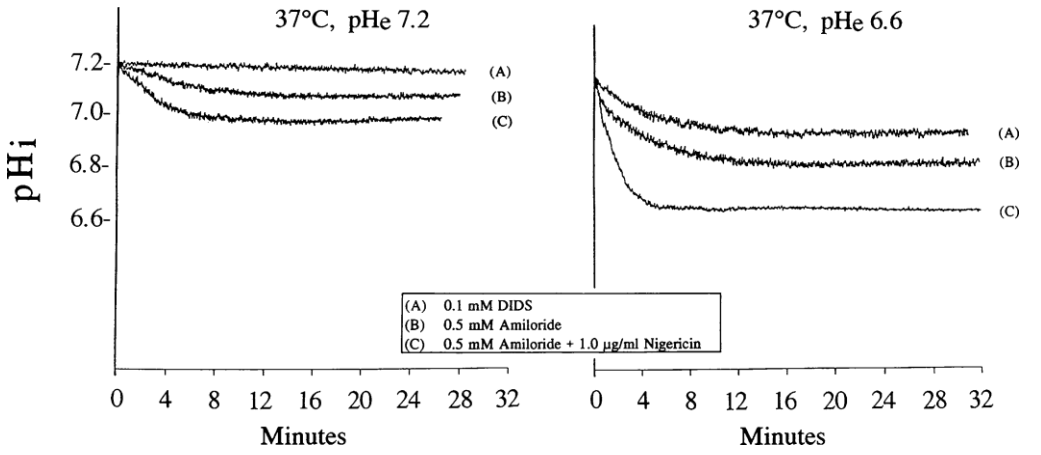

Fig. 5. Changes in pHi (BCECF intensity) in SCK tumor cells upon treating the cells with inhibi-tors of intracellular pH (pHi) regulatory mechanisms in extracellular pH (pHe) 7.2 or pHe 6.6. The decline in pHi caused by the inhibitors was much greater at pHe 6.6 than that at pHe.7.2.

cells in a less hostile environment. We have observed that when cells in culture were exposed to relatively mild acidic medium, cell cycle progression is slowed and thus, cell proliferation is slowed initially *(84)*. However, cells adapt eventually to the low-pH environment, and the proliferation rate is restored. It is conceivable that cells adapted to low pH are able to survive and form metastatic foci on distributing to other potentially suboptimal locations in the body.

5. Therapeutic Potential of Intracellular Acidification

Indications are that acidification of intracellular environment is cytotoxic to tumor
cells *(1,2,58,85–88)*. We have reported that the magnitude of decrease in pHi by inhibi-
tors of pHi regulation is significantly greater in an acidic pHe environment than in neutral
pHe environment. For instance, as shown in Fig. 5, a combination of amiloride, DIDS,
and nigericin reduced pHi of SCK tumor cells to 6.9 and 6.4 in pH 7.2 and pH 6.6 media,
respectively. Rotin et al. *(85)* reported that lowering pHi of tumor cells to 6.5 or lower
with nigericin, a K^+/H^+ ionophore, was cytotoxic. Inhibition of the Na^+/H^+ antiport with
amiloride or inhibition of the Cl^-/HCO_3^- exchange with DIDS alone was not toxic to the
cells, even when the pHe was as low as 6.0. However, combination of amiloride or DIDS

with nigericin was toxic to cells at pHe 6.5–6.8. Likewise, carbonylcyanide-3-chlorophenylhydrazine, which transports H^+ into cells, was toxic to tumor cells at pHe lower than 6.5, and its toxicity was greatly enhanced by amiloride or DIDS *(88)*. Apoptosis occurred in human leukemia HL-60 cells when pHi was lowered to 7.2–6.7 by inhibiting pHi regulation *(86,87)*. Increasing in intracellular Ca^{2+} with 4 *M* ionomycin, a Ca^{2+} ionophore, further increased the acid-induced apoptosis of HL-60 human leukemia cells. Importantly, the toxicity of various inhibitors of pHi regulation was observed to markedly increase when the cells were heated at 42–44 C *(1,2,9,11,89–97)*.

The direct mechanisms responsible for the cell death caused by low pHi is unclear. We have observed that an exposure of HL-60 human leukemia cells and other tumor cells to an acidic medium induces cell death through apoptosis of cells in G_1 phase *(86,87)*. The acid- induced apoptosis could be further increased when the pHi regulatory mechanisms were inhibited *(86)*. Detailed analysis indicated that a low- pHi environment first upregulates proapoptotic protein Bax, thereby activating caspases followed by poly(ADP-ribose) polymerase cleavage and DNA fragmentation *(87)*. Interestingly, exposing cells to pH 6.2 medium was less effective than exposing to pH 6.4 or pH 6.6 medium in causing apoptosis in HL - 60 cells *(87)*. It was concluded that there are optimal pH values for the major events in the apoptosis cascade such as Bax activation, caspase activation and activity, poly(ADP-ribose) polymerase cleavage, and DNA fragmentation so that an extremely acidic environment such as pH 6.2 was less effective than a pH 6.4–6.6 envi-ronment in inducing cell death via apoptosis. Recent studies *(98,99)* have indicated that cell death caused by certain chemotherapy drugs was attributable to an acidification of cells as a result of inhibition of pHi regulation mechanisms caused by H_2O_2 produced by mitochondria. These results demonstrate that the pHi regulatory mechanism may be an effective therapy target, because inhibition of pHi regulation will cause a reduction of

pHi preferentially in tumor cells in acidic extracellular environment relative to normal cells and thus cause damage preferentially in tumor cells.

6. Effect of pH on Radiation Damage

Unlike extensive studies on the effects of hypoxia on radiosensitivity in the past, little has been studied in regard to the effects of acidic pH on radiosensitivity. In a series of studies, we observed that acidic environments markedly prolong radiation-induced G_2 arrest in cancer cells *(84,100–103)*. For example, when RKO human colorectal cancer cells were irradiated with 12 Gy in pH 7.5 medium, the G_2 arrest peaked at 12–16 h, and then the cells progressed into G_1 phase or died of apoptosis. On the other hand, when RKO cells were irradiated with 12 Gy and maintained in pH 6.6 medium, significant portions of cells were still in G_2 arrest 72 h after irradiation (Fig. 6). Interestingly, the radiation-induced G_2 arrest in acidic pH medium rapidly decayed as soon as the acidic pH medium was replaced with neutral pH medium *(100)*. Importantly, the apoptosis and clonogenic cell death caused by irradiation were significantly less in acidic medium than in neutral pH medium (Fig. 7) . It appeared that the increase in radioresistance in acidic pH environment resulted from an increased DNA damage repair during the prolonged G_2 arrest. Similar increases in radioresistance in low extracellular pH environment have been reported by others *(104–106)*. Importantly, the environmental pH had to be reduced after treatment in order to confer resistance *(104)*.

Our studies indicate that the prolonged G_2 arrest after irradiation in an acidic pH medium was due, at least in part, to activation of CDC2, which is known to inhibit cyclin B1-CDC2 kinase activity responsible for the progression of cells through G_2/M phase *(101)*. Because the radiation-induced changes in cell cycle progression, apoptosis, and clonogenic cell death are intimately related to p53 expression, we have investigated the effect of pH on the kinetics of p53 expression *(107)*. We found that acidic environments significantly enhance the radiation- induced expression of p53, partly by increasing the formation of p53 and also partly by slowing down the degradation of p53 through inhi-bition of p53–murine double minute 2 (p53–MdM2) complex formation.

7. Effects Of pH On Hyperthermia Damage

It is well established that an acidic environment markedly increases thermal damage *(108–114)*. Detailed studies by a number of investigators using different cell lines dem-

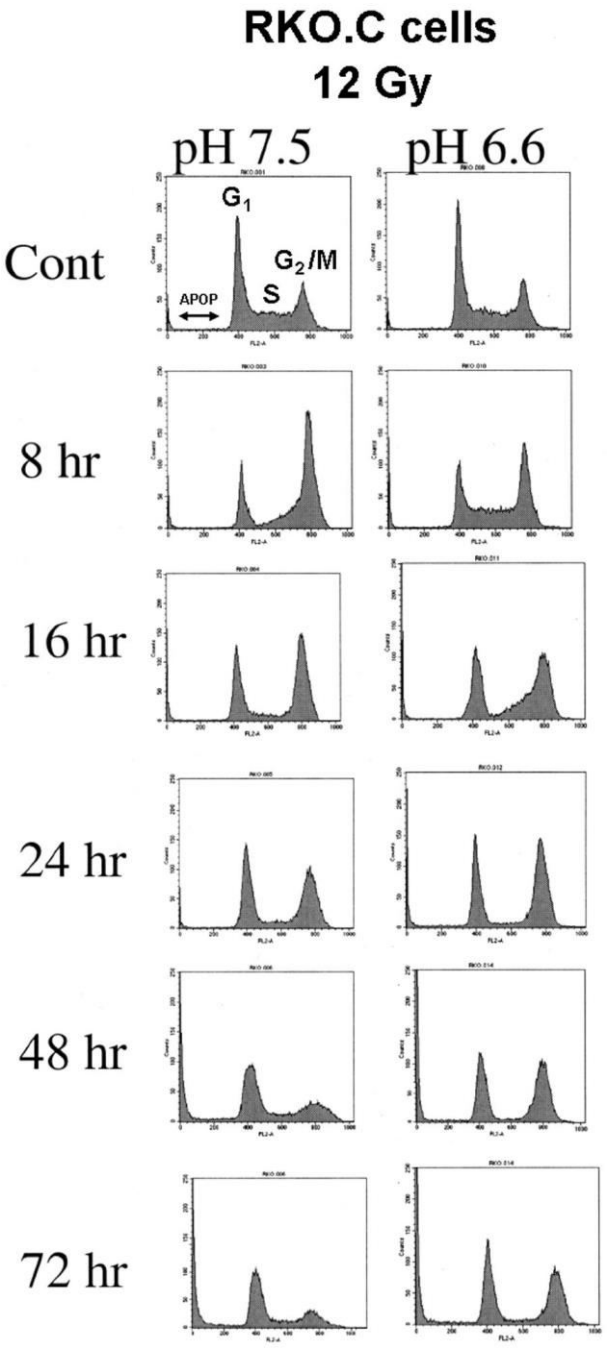
RKO.C cells
12 Gy
pH 7.5
pH 6.6
Cont
8 hr
16 hr
24 hr
48 hr
72 hr
G1
G2/M
S
APOP

Fig. 6. Cell cycle progression and apoptosis, as demonstrated with flow cytometry, of RKO human colorectal cancer cells after irradiation with 12 Gy in pH 7.5 or pH 6.6 medium. The cell cycle progression was delayed particularly at G_2/M phase after irradiation in pH 6.6 medium compared to the delay caused in pH 7.5 medium.

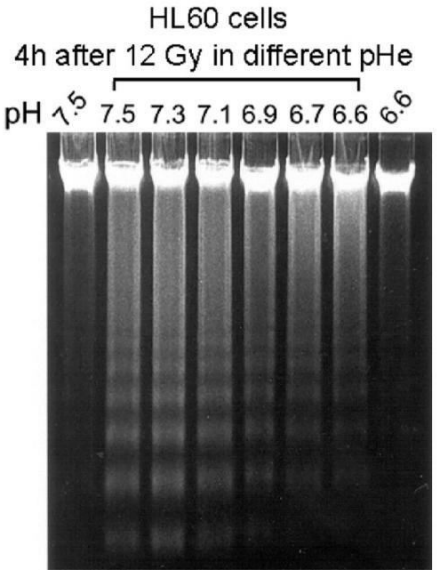

Fig. 7. Apoptotic fragmentation of DNA from HL- 60 cells 4 h after irradiation with 12 Gy in different pH media. The radiation-induced apoptosis was markedly suppressed as the medium pH was lowered.

onstrated that pHi, and not pHe, is the determinant of the thermosensitivity *(113,114)*. Hahn and Shiu *(115)* reported that Chinese hamster ovary cells maintained in acidic medium for prolonged periods were not as heat-sensitive as the cells exposed to acidic medium shortly before heating, and concluded that adaptation to a low-pH environment rendered the cells resistant to heat. Cook and Fox *(116)*, and Chu and Dewey *(114)* found that the pHi of the cells that adapted to a low pH environment was significantly higher than the pHi of the unadapted cells. Furthermore, the thermal survival curves of cells adapted and unadapted to a low-pH environment were identical when the survival curves were plotted against pHi instead of pHe. Chu and Dewey *(114)* therefore concluded that the increase in pHi was the reason why cells that adapted to a low- pH environment were

resistant to heat at low pHe relative to the unadapted cells. van der Berg et al. *(117)* reported that the thermosensitivity of human tumors showing an acidic interstitial pH was not necessarily greater than the thermosensitivity of tumors showing neutral interstitial pH. They concluded, therefore, that the cells in human tumors adapted to an acidic environment, and thus, the tumor cells were not heat-sensitive despite the low intratumor pH. Conceivably, an acute acid loading to cells adapted to a certain pHe would still render the areas heat-sensitive.

The intratumor pH has been observed to decrease markedly during heating of tumors, probably owing to vascular damage *(3,14,15,20,118)* and to the resultant increase in the accumulation of lactic acid *(119)*. Acute build-up of acidity during heating, particularly in the nonacidic areas, would be expected to sensitize tumor cells to heat. We observed that tumor cells in vivo were far more thermosensitive than the same cells in vitro, and we concluded that the acidic intratumor environment and the further increase in acidity during heating enhanced the thermal damage to the tumor cells *(14,119)*. The vasculature in human tumors has been reported to be more heat-resistant than the vasculature in rodent

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tumors, and thus, the pH in human tumors may not drop as severely as in animal tumors on heating *(32,120)*. However, it is also quite possible that the human tumors in these previous studies were not adequately heated, and thus, the blood flow as well as the tumor pH remained unchanged. It is likely that both in human tumors or animal tumors, the pH will decrease on application of hyperthermia if heating temperature is high enough to cause vascular damage and induce hypoxia. Along these lines, attempts are being made to sen-sitize human tumors to hyperthermia by acidifying the tumors using hyperglycemia *(121)*.

8. Effect of pH on Chemotherapy

It is known that the influx of drugs into tumor cells will be greatly affected by the pK_a value of the drugs. The acidic extracellular environment in tumors traps weakly basic drugs, thereby hindering the influx of the drugs into cells, whereas it enhances the influx of weakly acidic drugs into cells. Furthermore, the pH gradient between the vesicular compartments and the cytosol of cells has been known to be considerable. Therefore, it is conceivable that weakly basic drugs may be trapped inside the acidic compartments, thereby limiting their cytotoxicity *(122,123)* . It follows that cells containing a larger number of acidic vesicles may be resistant to weakly basic drugs, whereas they may be sensitive to weakly acidic drugs.

In addition, intracellular pH may affect the molecular interaction between drugs and their targets such as various intracellular organelles, DNA, RNA, proteins involved in cell cycle progression and cell division, and signals involved in apoptosis. The effect of pH on commonly used anticancer drugs is briefly addressed in this section.

8.1. At Normal Temperatures

Table 2 shows the relative cytotoxicity of various anticancer drugs in acidic (pH < 7.0), neutral (pH 7.0–7.4), and alkaline (pH >7.4) environments. The cytotoxicity of *bis*-chloroethylating agents such as cyclophosphamide and its derivatives, e.g. mafosfamide, nor-nitrogen mustard, melphalan, and chlorambucil, was reported to be significantly increased in acidic pH environments *(124,125)*. Cyclophosphamide is a prodrug, and a low-pH environment accelerated its bioactivation. On the other hand, the cytotoxicity of ifosfamide, an oxazaphosphorine analog of cyclophosphamide, was unaffected by the environmental acidity *(124)* . In ifosfamide, one of the chloroethryl side chains is shifted from an amino nitrogen to a ring nitrogen. Therefore, it was concluded that the *bis*-chloroethyl amine group may be a critical determinant for the H^+ ion-mediated enhance-ment of cytotoxicity in this group of agents *(124)*. The cytotoxicity of mafosfamide could be enhanced markedly by increasing intracellular acidity with nigericin (K^+/H^+ iono-phore) in acidic medium *(124,125)*. Jahde et al. *(124)* concluded that the increase in the cytotoxicity of cyclophospamide and its derivatives in an acidic pH environments were because of an increase in the cellular uptake of the drugs and also to an increase in the monofunctional alkalinization of DNA. It was further concluded that the phase of DNA crosslink formation and that of crosslink removal were relatively independent of the environmental pH. Skarsgard et al. *(126)* reported that a low-pH environment potentiated the cytotoxicity of melphalan and chlorambucil by increasing the uptake of the drugs. Methylmethane sulphonate, a monofunctional alkylate, was reported to be independent of environmental acidity *(115)*. The alkylating potency of *bis*-chloroethylnitrosurea (BCNU) was also independent of environmental acidity *(115)*, whereas that of cyclohexyl-chloroethylnitrosourea was reported to decrease in acidic environment *(127)*.

The cytotoxicity of thiotepa, another alkylating agent, increased when the environment was made acidic *(128)*. The cytotoxicity of both triethylenemelamine *(129)* and thiophosphamide *(130)*, alkylating agents, against transplanted rodent tumors was found to be increased when the intratumor environment was made acidic by induction of hyper-glycemic. On the other hand, the effect of methotrexate, an antimetabolite known to be very effective against certain cancers, was independent of pH in vitro *(131)*. 5-

Fluorou-racil is a prodrug and becomes an antimetabolite after intracellular conversion. 5-fluo-rouracil is a weak acid, and thus, acidic pH environment increases its cellular uptake *(132)*. Mitomycin C, bleomycin, amphotericin B, and doxorubicin (Adriamycin™) are naturally occurring anticancer agents. The cytotoxicity of mitomycin C, a bioreductive alkylating agent, slightly increased when the environmental pH was lowered *(133)*. The increase in mitomycin C cytotoxicity in an acidic environment appeared to be because of an increase in the DNA crosslinking. The cytotoxicity of bleomycin *(115,131,134)* and amphotericin B *(115,131,135)* was unchanged, whereas doxorubicine toxicity declined in an acidic pH environment *(134,136)*. Doxorubicin has a primary amine with a basic pK_a, and thus, its cellular uptake may be reduced in an acidic medium. Indeed, the uptake of doxorubicin at pH 6.6 environment was only one half of that at a pH 7.4 environment *(122,123)*. Furthermore, doxorubicin is trapped and sequestrated in acidic vesicles within the cytoplasm, which prevents the interaction of the drug with its target. A number of agents have been used to enhance the cytotoxicity of doxorubicin by inhibiting the for-mation of acidic vesicles, thereby releasing the doxorubicin into the cytoplasm *(122,123)*. Vinblastine and vincristine are also naturally occurring anticancer drugs. The uptake of these alkaloids has been reported to decline in an acidic environment *(137)*. The pK_a of vinblastine and vincristine are 5.0–5.5 and 7.4 at physiological pH, respectively. We have reported previously that intracellular acidification alone is able to activate caspases, thereby triggering apoptosis *(86,87)*. Interestingly, apoptosis in cancer cells caused by certain chemotherapy drugs has been attributed to intracellular acidosis caused by the drugs. As mentioned previously, Hirpara et al. *(98)* reported recently that chemotherapy drugs trigger production of H_2O_2 by mitochondria, which then inhibit the Na^+/H^+ ex-changer, resulting in intracellular acidification. The resultant intracellular acidification causes mitochondrial recruitment of Bax and release of cytochrome *c* from mitochondria, thereby activating the caspase cascade leading to apoptosis *(98,99)*. Lastly, paclitaxel is one of the taxanes extracted from yew trees and a common chemotherapeutic. It is highly lipophilic and devoid of any ionizable groups, with pK_a values in the physiological range *(139)*. Therefore, the cellular uptake of this drug is independent of pHe. In all, these results clearly indicate that efficacy of many, but not all, anticancer drugs may be signifi-cantly increased by altering intratumor pH based on the pK_a value of the drugs.

8.2. At Elevated Temperatures

Although the effect of methylmethane sulphonate *(115)*, BCNU *(115,131)*, methotr-exate *(137)*, bleomycin *(115,131,134)*, and amphotericin B *(4,115,131)*

were indepen-dent of the environmental pH at 37□C, their cytotoxicity increased in a low-pH environment if the cells were heated (*see* Table 2). Interestingly, the cytotoxicity of amphotericin B also increased when the environment was made alkaline at elevated temperatures *(4,115)*. Hahn *(138)* suggested that heat may increase the cellular uptake of certain drugs or inhibit the repair of damage caused by drugs, and the acidic environment accentuates these processes. Related to this, Hahn and Shiu *(115)* reported that the low-pH-adapted cells were resistant to thermochemotherapy with bleomycin, amphotericin B, and cisplatin, but not with BCNU. Thus, it was concluded that the pH dependence of cytotoxicity for some drugs at elevated temperature is affected by the pH history of the target cells.

Cisplatin is platinum complex with potent anticancer activity. The cytotoxicity of this drug increases with an increase in the environmental acidity *(115,139,140)*. Herman et al. *(84)* demonstrated that heating caused a greater increase in the cytotoxicity of cisplatin in an acidic pH environment than in a neutral pH environment. At 37□C and pHe 7.4, no difference in the sensitivity to cisplatin was observed between oxic cells and hypoxic cells. When cells were heated in pH 7.4 medium, the sensitivity of oxic cells to cisplatin mark-edly increased, whereas that of hypoxic cells remained unchanged. On the other hand, in pH 6.45 medium, the sensitivity of both oxic and hypoxic cells to cisplatin increased on heating. Herman et al. *(139)* also studied the cytotoxicity of analogs of cisplatin such as (1,2-diamino-4-nitrobenzene)dichloroplatinum(II) (Plato) and *trans -bis*-(2 -amino-5-ni-tro-thiazole)dichloroplatinum(II) (Plant) under various conditions. When the environ-mental acidity was increased, the cytotoxicity of Plato decreased and that of Plant increased. Unlike cisplatin, Plato and Plant were more toxic toward hypoxic cells than oxic cells, but the cytotoxicity of these drugs did not increase with an increase in tempera-ture. Teicher et al. *(140)* reported that the cytotoxicity of PtCl4(Fast Black)$_2$, an analog of cisplatin, was greater in an acidic than in a neutral environment at 37□C, and heating increased the cytotoxicity of this drug in both acidic and neutral pH environments. Oxic cells and hypoxic cells were equally sensitive to this drug at 37□C. However, when heated, oxic cells were slightly more sensitive to this drug in pH 7.4 medium, whereas hypoxic cells were slightly more sensitive to this drug in pH 6.45 medium. Teicher et al. *(141)* observed that the changes in the concentration of cisplatin and $PtCl_4$(Fast Black)$_2$ in the cells after the environmental pH and temperature were changed did not correlate with the changes in the cytotoxicity, and concluded that an increase in the reaction of the drugs with DNA was the direct cause of the increase in the cytotoxicity of the drugs in a low-pH medium at elevated temperatures. It was also suggested that metabolic changes that must occur to maintain neutral pHi in acidic environment may

increase directly or indirectly the response of the cells in an acidic environment to the drugs.

9. Acidification and Alkalinization of Tumors

Because an acidic intratumor environment increases the response of tumors to certain chemotherapeutic drugs and also to hyperthermic treatment, various attempts have been made to acidify the intratumor environment. It has long been known that tumors can be acidified by induction of hyperglycemia by administration of excess glucose *(10,142– 144)*. It was initially proposed that the decline in the intratumor pH by hyperglycemia resulted from an increase in glucose metabolism by aerobic glycolysis *(144)*. However, indications are that the decline in intratumor pH by hyperglycemia results not only from an increase in aerobic glycolysis, but also from an increase in anaerobic glycolysis as a consequence of blood flow decline and ensuing hypoxia. The mechanisms for the decline in tumor blood flow by hyperglycemia are complicated. A serious problem in using hyperglycemia for induction of acidosis in human tumors is that tumor acidification requires a large dose of glucose exceeding the tolerable level for most patients. Further-more, the reduction in blood flow by hyperglycemia may decrease the drug delivery to tumor cells. Acidification of rodent tumors by hyperglycemia could be enhanced by

concomitant administration of metaiodobenzylguanidine *(82,121)*, which inhibits mito-chondrial respiration at complex I of the electron transport chain, resulting in an increase in lactic acid formation.

Hydralazine, a vasodilator, also decreases tumor blood flow *(145–147)*, and thus, it may increase tumor acidity. As with hyperglycemia, tumor acidification by hydralazine may not be useful to enhance the effects of drugs, because drug delivery to tumors will be reduced owing to the decrease in tumor blood flow that occurs. Furthermore, the effect of hydralazine is strongly dependent on the location of the tumor in the body, and it can reduce blood flow in many normal tissues as well *(146,147)*.

Conversely, the cellular uptake of weakly basic chemotherapy drugs may be enhanced if pHe is raised to alkaline range. Indeed, treatment of tumor-bearing mice with sodium bicarbonate has been demonstrated to cause tumor-specific alkalinization of extracellular pH and increase the antitumor effect of the weakly basic drug, mitoxantrone, which has two ionizable amines with pK_a values of 8.3–

8.6 *(123)*. This strategy appears to have limited use because of the dangers of affecting blood chemistry and pH with buffering agents.

10. Conclusion

The intratumor environment is acidic because of elevated production of lactic acid and other acidic metabolities as a result of high aerobic and anaerobic glycolysis. However, the pHi of tumor cells is maintained at neutral range despite the acidic pHe by virtue of powerful pHi regulatory mechanisms. Lowering the pHi by inhibitors of pHi regulation is cytotoxic, particularly in a low-pHe environment. The acidic pHe and the gradient between pHe and pHi greatly affect the response of tumor cells to chemotherapy drugs, radiotherapy, and hyperthermia. The feasibility of controlling pHe and pHi by various means with the goal of increasing the response of tumor cells to various treatments is being investigated.

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