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Mini-review

Autophagy and cancer: Dynamism of the metabolism of tumor cells and tissues

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ABSTRACT

Autophagy is a dynamic process involving the bulk degradation of cytoplasmic organelles and proteins. Based on the function of "cellular recycling", autophagy plays key roles in the quality control of cellular components as well as supplying nutrients and materials for newly constructed structures in cells under metabolic stresses. The physiological relevance of autophagy in tumor formation and progression is still controversial. The cytoprotective function of autophagy in cells subjected to starvation might enhance the prolonged survival of tumor cells that are often exposed to metabolic stresses *in vivo*. Meanwhile, a tumor-suppressive function of autophagy has also been suggested. Autophagy-related cell death has been regarded as a primary mechanism for tumor suppression. In addition, the loss of autophagy induced genome instability and significant necrosis with inflammation in transplanted mouse tumor models, suggesting an additional function of autophagy in the suppression of tumor formation and growth. Until now, investigations supporting and proving the above possibilities have not been fully completed using clinical samples and equivalent animal models. Though monitoring and the interpretation of autophagy dynamism in tumor tissues are still technically difficult, identifying the autophagic activity in clinical samples might be necessary to clarify the pathophysiological relevance of autophagy in tumor formation and progression as well as to develop new therapeutic strategies based on the regulation of autophagy.

1. Introduction – an overview of autophagy

Autophagy (from the Greek "auto", meaning oneself, and "phagy", meaning to eat) refers to a process in which cytoplasmic components are delivered to the lysosome for bulk degradation. Three types of autophagy – macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) – have been identified. These processes differ in the mode of delivery to lysosomes. Microautophagy involves the direct sequestration of cytosolic components by lysosomes. In CMA, a cytosolic and lysosomal chaperone protein, hsc70, assists unfolded proteins to translocate into lysosomes. In this review, we will focus on the most widely investigated process: macroautophagy (herein referred to as autophagy) [1–5].

Autophagy is a conserved catabolic process that involves the sequestration of organelles and long-lived proteins residing in the cytoplasm into a unique organelle, the autophagosome. As shown in Fig. 1, an isolation membrane (also called a "phagophore") which is a double membrane consisting two parallel lipid bi-layers is formed and elongated. Isolation membranes start sequestrating cytoplasmic constituents in the first step of autophagy. Once the edges of the isolation membrane are fused, it becomes a unique lipid bi-layer vesicular organelle, the autophagosome. Sequestrated cytoplasmic components are completely engulfed by the autophagosome. Next, the autophagosome undergo a maturation process, in which the autophagosome fuses with early and late endosomes.

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Finally, maturated autophagosomes fuse with the lysosome. The engulfed components, as well as the inner membrane of the autophagosome, are degraded by lysosomal hydrolases such as cathepsins. Autophagic processes have been well characterized in yeast, and more than 30 autophagy-related genes (ATG) that encode the proteins executing autophagy have been identified in the field of yeast genetics [6]. Similar autophagic machineries have been observed in mammalian cells, and mammalian orthologs for ATGs and other autophagy regulating molecules have also been identified [7]. The detailed molecular events involved in these processes are described in another comprehensive review [8].

Compared with the ubiquitin–proteasome system, which recognizes specific targets for degradation, autophagy has been thought to engulf cytoplasmic constituents non-selectively. Recently, selective sequestration targeting specific organelles or invasive microbes has also attracted attention [9].

A very concise description of the function of autophagy is recycling; reducing waste and yielding resources. Damaged organelles and misfolded proteins accumulate in senescent cells or cells under various stresses, such as oxidative stress and infection. The autophagic machinery degrades and reduces this cellular "garbage". This function is particularly effective in postmitotic cells, like neurons and cardiac myocytes, since these cells cannot dilute such superfluous components by undergoing cell division. Basal autophagy is constitutively observed in these postmitotic cells and may have an important function for the quality control of cellular components [10]. On the other hand, self-digested components provide nutrients and materials for newly constructed cellular structures. The amino acids and fatty acids generated by autophagic degradation are used by the tricarboxylic acid (TCA) cycle to produce ATP, a main energy source for various cellular events. Methylpyruvate, a membrane-permeable derivative of pyruvate that serves as a substrate for the TCA cycle, restored ATP production in autophagy-deficient cells. Furthermore, supplementation with methylpyruvate rescued these cells from metabolic stress-induced cell death [11].

Observations of clinical samples and animal and cellular models have suggested a variety of physiological and pathological roles of autophagy, such as development, aging, host defense system, neurodegenerative diseases, muscle and cardiac diseases and cancer [12,13]. However, the relevance of autophagy in tumor formation and progression is still controversial. Although previous findings strongly suggest that autophagy contributes to sustainable cell survival, anti-tumorigenic roles of autophagy have been also mentioned. Here, we will summarize and discuss recent studies of autophagy in cancer biology with the goal of clarifying this issue.

2. Autophagy protects cancer cells from starvation

Cancer cells in solid tumors obtain their necessary nutrients from blood flow. Aberrantly proliferating cancer cells may have high bioenergetic demands and require more nutrients than non-cancerous cells. Tumor angiogenesis is a reasonable way to increase blood flow. During the initial phase of tumorigenesis, tumor vessels have not yet been induced and the nutritional demands of tumor cells are likely to surpass the supply from normal vasculature. Moreover, even after tumor vessels have been established, the oxygen tension and glucose concentration in locally advanced tumors remain at a low level. This suggests that the tumor microvasculature is functionally and structurally immature to support sufficient blood flow [14–16]. Or, even once functionally and structurally adequate tumor vessels were established, soon or later, the balance of supply and demand would be ruined by disorganized tumor cell proliferation resulting in the disastrous dysfunction of the tumor vessels. The reduction of functional blood flow is significant in clinically hypovascular tumors such as pancreatic cancers [17]. Under these conditions, cancer cells are likely to encounter chronic ischemia leading to a shortage of nutrients. Cancer cells might adapt themselves to such a harsh microenvironment. In experimental cell culture systems, several cancer cells were resistant to nutrient-deprived conditions. For example, several pancreatic cancer- and colorectal cancer-derived cell lines showed a survival rate of more than 50% after 48 h of culture in a medium completely lacking carbon and nitrogen sources. Contrastingly, non-transformed fibroblasts were completely abolished within a day under the same conditions [18]. These findings suggest that cancer cells use alternative metabolic processes for their survival under starved conditions.

Metabolic stresses reportedly induce apoptosis [19]. In many tumor cells, apoptosis is suppressed by the overexpression of anti-apoptotic molecules or by the lack of pro-apoptotic molecules. Less apoptosis may explain the limited death of cancer cells under metabolic stresses, but the manner in which cancer cells obtain their necessary nutrients remains a mystery. One possible solution is that the cells digest their own components and obtain amino acids as an alternative energy source. Autophagy seems ideal for cancer cells to maintain energy homeostasis in such an autonomous fashion. Autophagy is known to be induced by different forms of metabolic stress, including nutrient deprivation, growth factor deprivation, and hypoxia [13,20]. Since these conditions are often observed in physiological tumor microenvironments, autophagy is likely activated in cancer cells.

We should also remind the apparent discrepancy between the tumor "cell" doubling time and the tumor "volume" doubling time. The potential tumor cell doubling time estimated by *in vivo* measurement of S-phase duration was remarkably rapid in many solid tumors, a median value of the order of 5 days. However, tumor volume doubling time determined by radiological measurement of the size of these tumors was much slower, months or years [21,22]. It suggests that about half of the proliferating cancer cells are subsequently lost under tumor microenvironment though many of them have already acquired anti-apoptotic function. Is there any physiological relevance of such dynamism of cell kinetics? A potential scenario is that the vanishing cells serve nutritional sources for surrounding surviving cells. In this sense, inducing cell death and supporting cell survival under metabolic stresses are not

contradicting. Autophagy is also known to induce cell death and possibly sacrifice some part of individual tumor cell for supporting a prolonged survival and continuous growth of other cells in the tumor mass.

Several experimental methods have been used to identify the activation of autophagy (Table 1) [23]. Measuring the turnover of long-lived proteins provides a biochemical clue to autophagy. Transmission electron microscopy reveals the ultrastructure characteristics of the autophagosome. Autophagosome formation is also conveniently monitored by following a phosphatidylethanolamine (PE)-conjugated form of yeast Atg8 or mammalian LC3, one of the mammalian orthologs of Atg8. During autophagy, LC3 shifts from a soluble form to a membrane-bound form (LC3-II) and is incorporated into the autophagosomal inner membrane. The existence of autophagosome-incorporated LC3-II can be detected biochemically (immunoblotting against LC3) or microscopically (immunocytochemistry against LC3 or exogenously expressed, fluorescently tagged LC3).

The ultrastructure of autophagosomes in tumor cells has been observed in several experimental systems, including a rat pancreatic cancer model [24]. A high potential for autophagic protein degradation was observed in an undifferentiated colon cancer cell line, HT29, and other transformed cells [25]. Nutrient deprivation-induced LC3-II turnover was observed in colorectal cancer-derived cell lines that showed resistance to starvation [26]. In addition to these findings in cultured cancer cells, the increased expression of autophagy-related proteins, including BNIP3 and LC3, was observed specifically in colorectal and gastric cancer epithelia in surgically-resected specimens [26,27].

Increasing evidence implies that autophagy has a cytoprotective role in cancer cells under metabolic stress. Transplanted epithelial tumors in which the Beclin 1 or Atg5 alleles were deleted showed a reduction in autophagy and an increase in cell death in regions exposed to metabolic stress [28–30]. Similarly, a cytoprotective function of autophagy was observed in cultured cancer cells. The genetic inactivation of autophagy by the suppression of ATG expression using RNA interference or the constitutive activation of PI3K induced cell death in response to metabolic stresses [28–30]. RNA interference of ATG5, Beclin 1 and ATG7 enhanced tamoxifen-induced apoptosis in tamoxifen-resistant breast cancer cell lines [31]. ATG5 knockdown also enhanced the effect of alkylating drug-induced cell death [32]. The pharmacological inhibition of autophagy also induced nutrient deprivation-induced cell death [26]. Chloroquine, which interferes with lysosomal function, inhibited autophagy and suppressed Myc-induced lymphomagenesis in a transgenic mouse model [32].

Though the above findings suggest that autophagy may contribute to tumor cell survival and tumor formation, the molecular mechanisms underlying the acquisition of vigorous autophagic activity in cancer cells have remained unclear. Recently, Kroemer and his colleagues reported a potential anti-autophagic function of cytoplasm-localizing p53 [33]. They observed the degradation of p53 under metabolic stresses followed by the induction of autophagy. Contrastingly, the loss of p53 resulted in the consistent activation of autophagy in a series of cell lines. While overexpression of wild-type p53 reduced the aberrant autophagosome formation, a mutant p53 protein which harbors a codon 175 mutation (R175H), which is frequently identified in clinical human cancers, did not affect the higher basal autophagic activity of p53-null cells. Though most of their observation was limited in the culture cell system, further studies which clarify its relevance to tumor formation and progression are awaited.

3. Autophagy and tumor suppression

An opposite perspective was presented by a study examining a Bcl-2-binding protein, Beclin 1. Levine and

Table 1
Recommanded methods for monitoring autophagy in higher eukaryotes [23].

Criteria	Methods
Monitoring phagophore and autophagosome formation by steady state methods	
1. Electron microscopy (increase in autophagosome quantity)	Quantitative electron microscopy, immunoelectron microscopy
2. Atg8/LC3 Western blotting and ubiquitin-like protein conjugation systems (increase in the amount of LC3-II, and Atg12–Atg5 conjugation)	Western blot
3. Fluorescence microscopy (increase in punctate LC3 (or Atg18))	Fluorescence, immunofluorescence and immunoelectron microscopy
4. TOR and Atg1 kinase activity	Western blot, immunoprecipitation or kinase assays
5. Transcriptional regulation	Northern blot or qRT-PCR
Monitoring autophagy by flux measurements	
1. Autophagic protein degradation	Turnover of long-lived proteins
2. Turnover of LC3-II	Western blot +/− lysosomal fusion or degradation inhibitors
3. GFP-Atg8/LC3 lysosomal delivery, and proteolysis (to generate free GFP)	Fluorescence microscopy, FACS Western blot +/− lysosomal fusion or degradation inhibitors
4. p62 Western blot	Western blot with qRT-PCR or Northern blot to assess transcription
5. Autophagic sequestration assays	Lysosomal accumulation by biochemical or multilabel fluorescence techniques
6. Turnover of autophagic compartments	Electron microscopy morphometry/stereology
7. Autophagosome-lysosome colocalization and dequenching assay	Fluorescence microscopy
8. Sequestration and processing assays in plants	Chimeric RFP fluorescence and processing, light and electron microscopy
9. Tandem mRFP-GFP fluorescence microscopy	Fluorescence microscopy of tandem mRFP-GFP-LC3
10. Tissue fractionation	Centrifugation, Western blot and electron microscopy
11. Analyses *in vivo*	Fluorescence microscopy and immunohistochemistry

her colleagues first identified Beclin 1, a mammalian ortholog of ATG6, as a candidate tumor suppressor. Beclin 1 interacts with class III PI3-kinase, Vps34, and this interaction was crucial for the induction of autophagy and suppression of the growth of the xenografted breast cancer cell lines [34–36]. Vps34 is also required for normal protein trafficking pathways such as the delivery of proteases from the trans-Golgi network to the lysosomes. Atg6 (Vsp30) was reportedly involved in the regulation of both autophagy and endosomal membrane trafficking in yeast. Whether mammalian Beclin 1 takes part in the membrane trafficking is still controversial [34,35].

The hemiallelic loss of the Beclin 1 coding gene was observed in 40–75% of sporadic human cancers in the breast, ovary and prostate [37]. A gene-targeted mouse model of Beclin 1 provided another clue [38,39]. The homozygous deletion of Beclin 1 led to embryonic lethality. Meanwhile, Beclin 1 heterozygous mutant mice showed decreased autophagy and increased spontaneous tumors, including lung and liver cancers and lymphomas. In these mice, no loss of heterozygosity (LOH) was observed and the remaining wild-type allele of Beclin 1 was intact. Furthermore, Beclin 1 protein expression was reduced but not completely diminished in mouse tumors as well as human clinical samples [36]. These findings suggest that Beclin 1 is a haplo-insufficient tumor-suppressor gene. The tumor-suppressive function of Beclin 1 was reinforced by the relevance of Beclin 1-associated proteins to tumor suppression. UVRAG was initially identified from the cDNA library, which partially rescued the UV sensitivity of a xeroderma pigmentosum (XP) cell line. The screening of Beclin 1-binding proteins revealed that UVRAG was recruited to the Beclin 1–class III PI3K complex. UVRAG activated Beclin 1 and induced autophagosome formation [40]. In addition, UVRAG is involved in autophagosome maturation. UVRAG interacts with class C tethering proteins (Vps11, Vps16, Vps18 and Vps33) resulting in activation of a small GTPase, Rab7 which enhances the fusion of autophagosomes to endosomes [41]. As seen with Beclin 1, UVRAG is monoallelically mutated in human colorectal cancers [40]. Bif-1 (also known as Endophilin B1) interacts with Beclin 1 through UVRAG. Bif-1 activated class III PI3K and induced autophagy. Spontaneous tumor formation was increased in Bif-1 deficient mice [42].

During investigations of autophagy in mammalian cells, several signaling pathways have been revealed to regulate autophagy. Interestingly, correlations between pro-autophagic molecules and tumor suppressors and between anti-autophagic molecules and oncogene products can be pointed out (Fig. 1). The mammalian target of rapamycin (mTOR) is a key molecule for regulating cancer cell proliferation. Rapamycin inhibits mTOR function followed by autophagy induction [11,43]. Molecules known to suppress mTOR, including PTEN and TSC, both of which are regarded as tumor-suppressor gene products, induce autophagy [44,45]. Meanwhile, mTOR-activating molecules like class I PI3K and Akt, which are frequently activated in various cancer cells, inhibit autophagy [11,46]. The involvement of p53 in the activation of autophagy has also been suggested. In contrast to the inhibitory effect of cytoplasmic p53 men-

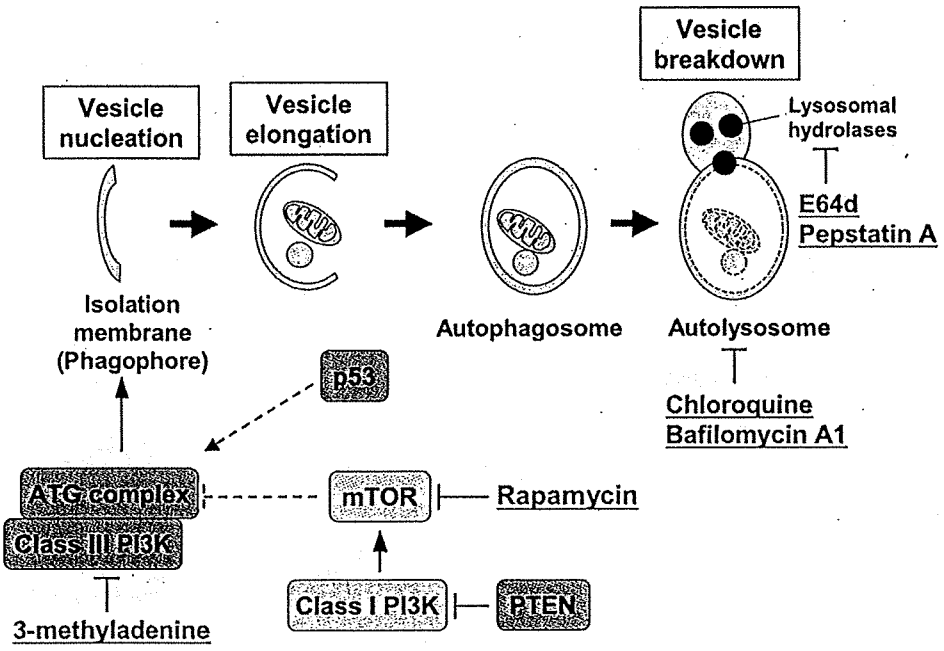

Fig. 1. Cellular events during autophagy (macroautophagy). In the first step of autophagy, the isolation membrane (phagophore) is elongated and sequestrates the cytoplasmic constituents. Fusion of the edges of the isolation membrane forms an autophagosome, which contains the sequestrated components. A lysosome then fuses with the autophagosome, and lysosomal hydrolases degrade the engulfed components along with the inner membrane of the autophagosome. Mammalian homologues of ATG (autophagy-related genes) have been identified. Class I PI3K and mTOR signaling inhibits autophagy activation, whereas tumor-suppressor proteins like p53 and PTEN induce autophagy. Rapamycin inhibits mTOR and induces autophagy. 3-Methyladenine, chloroquine, bafilomycin A1, E64d and pepstatin A inhibit autophagy at different points. These small molecules are used to inhibit autophagy in experimental model systems.

tioned above, the transactivating function of nuclear p53 takes a role in the autophagy induction. DRAM is a direct transcriptional target of p53, and its product localizes in the lysosomal membrane. The activation of p53 induced an increase in autophagy in a DRAM-dependent manner [47]. p53 was also reported to affect autophagy via the suppression of mTOR [45]. DAPk, death-associated protein kinase, induces autophagy as well as apoptosis. The tumor-suppressive function of DAPk has also been described, and promoter hypermethylation of the DAPk locus has been reported in several human cancers [48].

These findings suggest the likely relevance of autophagy to tumor suppression. However, the above-mentioned pro- and anti-autophagic molecules, like p53 and mTOR, are pluripotent proteins that regulate cell proliferation and death via various molecular events. The net contribution of autophagy to cell fate decisions during tumorigenesis should be carefully estimated, but this objective has not yet been successfully accomplished.

4. Autophagy and cell death

Autophagy has attracted much attention in connection with cell death. "Type II" or "autophagic" non-apoptotic programmed cell death is morphologically defined by the existence of autophagosomes [49]. Autophagic death has been reported in cancer cells, especially those that have been treated with chemotherapeutic or radiotherapeutic agents [50]. This cytotoxic effect has been supposed to be the main reason for the tumor-suppressive function of autophagy. Self-cannibalism, that is, the situation in which excess autophagic catabolism exceeds the capacity for cellular anabolism, is frequently argued as a potential mechanism for autophagic death. Though autophagy has been regarded to degrade cytoplasmic constituents non-specifically, the specific autophagic degradation of target proteins and organelles has also been reported. The specific degradation of cytoprotective factors is another undeniable mechanism of autophagic death [51]. However, experimental evidence supporting these ideas has not yet been obtained. Moreover, cytotoxic stimuli, such as oxidative stress, induces both autophagy and cell death, but whether autophagy is an active death-inducing mechanism (cell death by autophagy) or a result of an unsuccessful effort to prolong the survival of damaged cells (cell death with autophagy) is difficult to distinguish.

Recent findings have raised a question about the relevance of autophagic death to tumor suppression. The allelic loss of Beclin 1 inhibited both basal and stress-induced autophagy in immortalized baby mouse kidney epithelial cells. Metabolic stress-induced cell death was apparently increased, suggesting loss of autophagy led impaired cytoprotective function. Despite impairment in autophagy-mediated cell survival, Beclin 1+/− cells were more tumorigenic than the wild-type control cells [28]. To make a plausible explanation for these contradictory findings, the loss of cytoprotection induces tumorigenesis, is still challenging.

5. Potential mechanisms for the tumor-suppressive function of autophagy

Recently, White and colleagues have proposed interesting hypotheses about the above issues (Fig. 2). Under meta-

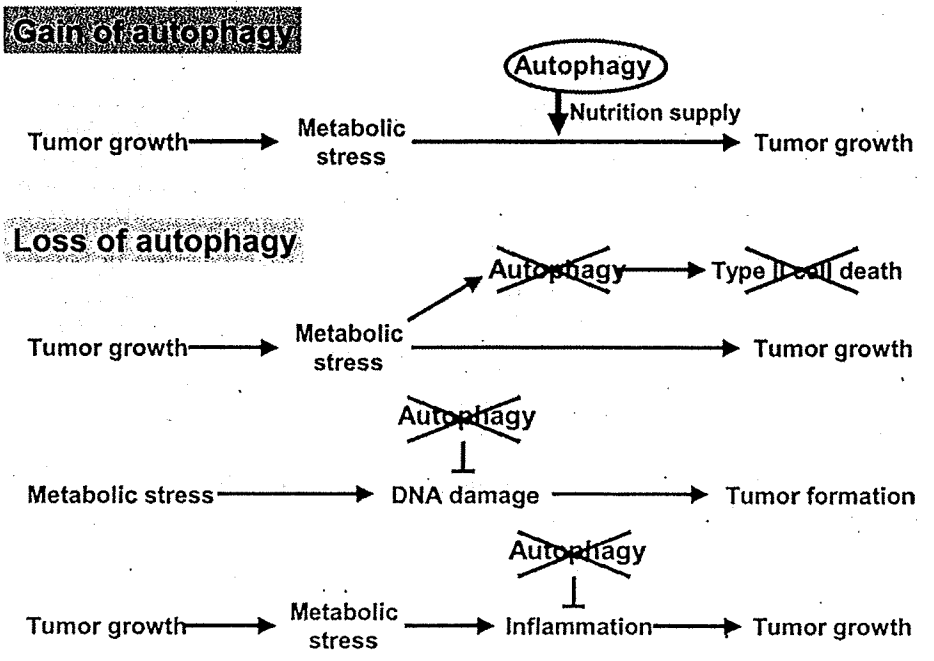

Fig. 2. Both the gain and loss of autophagy are possibly involved in tumor formation and growth. The activation of autophagy supplies nutrients to tumor cells under metabolic stress. On the other hand, the eradication of tumor cells by autophagy-related cell death is assumed to occur, and the loss of autophagy may lead to tumor cell survival. A recent mouse model using transplanted autophagy-deficient tumor cells revealed that the loss of autophagy induced DNA damage and inflammation, enhancing tumor formation and growth.

bolic stress, Beclin 1+/– epithelial cells overexpressing anti-apoptotic Bcl-2 showed an increase in DNA double strand breaks, gene amplification, and chromosomal number disorder. They assumed that the loss of autophagy induces DNA damage and chromosomal instability, followed by increased tumor susceptibility [30]. The exact mechanisms for how autophagy maintains genome stability remain unclear. One possibility is that cells lacking autophagy are unable to reduce damaged mitochondria and peroxisomes. These damaged organelles are potential sources of reactive oxygen species that induce genotoxic stress.

When the same autophagy-deficient epithelial cells were exposed to starvation, apparent necrotic cell death was observed when apoptosis was inhibited. Furthermore, significant inflammation was induced in the tumor tissues in which autophagy-deficient cells were transplanted [28]. Necrosis is often associated with macrophage infiltration *in vivo*, and tumor-associated macrophages enhance tumor progression [52]. It is an interesting hypothesis that the inflammation induced by a lack of autophagy may be correlated with tumor progression *in vivo*.

The above scenarios are not incompatible with the idea that autophagy protects cancer cells from metabolic stress-induced death, and these potential mechanisms are worthy of further discussion to elucidate the physiological roles of autophagy in tumorigenesis.

6. Perspective – remaining tasks

The relevance of autophagy to cancer remains a frustrating topic to discuss. It is necessary to understand the consequences of the loss or gain of autophagy in the context of the tumor microenvironment. Previously proposed hypotheses for how autophagy contributes to tumor biology, either pro- or anti-tumorigenic, are possible and interesting, but most of them have arisen from artificial experimental systems and no direct clinico-pathological evidence supports these ideas. Evaluating autophagy in clinical tumor samples has been difficult, mainly because of the lack of appropriate markers for detecting active autophagy. Autophagosome formation in tumor tissues has been morphologically confirmed using electron microscopy, but this method is not suitable for handling a large number of specimens. Recently, anti-LC3 antibodies for immunostaining have become available [26,53,54]. LC3-II proteins incorporated into autophagosome membranes exhibit a punctuate cytoplasmic staining pattern during active autophagy. LC3 immunostaining is an easier method of evaluating autophagosome formation in conventional formaldehyde-fixed surgically-resected specimens. We applied polyclonal anti-LC3 antibody for the immunostaining of colorectal cancer specimens. In over 90% of the cases, LC3 accumulated specifically in cancerous epithelia but not in adjacent non-cancerous mucosa [26]. Similar cancer-specific accumulation of LC3 was observed in pancreatic cancer. Interestingly, strong LC3 expression in the peripheral area of cancer tissue was correlated with a poor prognosis (Fig. 3) [55]. As we mentioned earlier, both pancreatic and colorectal cancer-derived cell lines are often resistant to the nutrient-starvation. It seems more than coincidence that specific LC3 accumulation was observed in the clinical samples of these tumors. Further investigation using various cancer tissue samples may reveal the contribution of the gain or loss of autophagy in tumor formation and progression more clearly.

Autophagy is executed by dynamic and multiple cellular processes, including the formation of the autophagosome, the delivery of cytoplasmic constituents to the lysosome, and the digestion and recycling of these target molecules and organelles. For the precise evaluation of autophagic activity, the "autophagic flux" must be estimated. For example, the accumulation of autophagosomes reflects either an increase in autophagosome formation (activation of autophagy) or a reduction in the degradation of autophagosomes (inhibition of autophagy). To determine the flux, the rate of long-lived protein degradation must be measured or the changes in appropriate autophagy markers, such as the LC3-II protein level, must be assessed with or without the arrest of autophagic flux at a given point of blockage. Recently, Klionsky and 231 other scientists published guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes (Table 1) [23]. In these guidelines, the authors strongly recommended the measurement of autophagic flux for monitoring autophagy. Detecting autophagosome formation using steady state methods, including electron microscopy or LC3 immunostaining, should be combined with flux measurements. However, applying such dynamic assays to clinical samples is often technically difficult.

The above-mentioned guidelines do not provide a full resolution of the above difficulties. Further discussion might be needed to establish standard methods for evaluating autophagy in clinical samples. To assess autophagic activity using steady state methods, not only one parameter, but multiple autophagy-related molecules should be applied. For example, while the loss of Beclin 1 implies a reduction in autophagosome formation, this outcome should be confirmed by a decrease in autophagosome-specific markers, such as LC3-II. Mimicking *in vivo* events on *ex vivo* systems may support the findings obtained in clinical samples using steady state methods. In such cases, it should be remembered that autophagy is induced under complex microenvironments in cancer tissues. As mentioned previously, cancer cells are often exposed to chronic ischemia. Under this situation, not only nutrients but also oxygen, growth factors and other components provided by blood flow might be decreased. Meanwhile, metabolites and other components excreted from cancer cells may not be properly removed from the cancer tissues. Cancer cells are also directly and indirectly influenced by surrounding stromal cells and extracellular matrices. Reconstituting all these factors in an experimental cell culture system is practically unfeasible, but the above restrictions should always be taken into account when evaluating the results obtained from any model system.

To analyze autophagy function under more physiological conditions, appropriate animal models are eagerly needed. Xenografted and/or isografted animals in which the transplanted transformed cells lacking autophagy-related genes are useful, but not perfect. Transplanted tumors do not always reproduce the microenvironmental

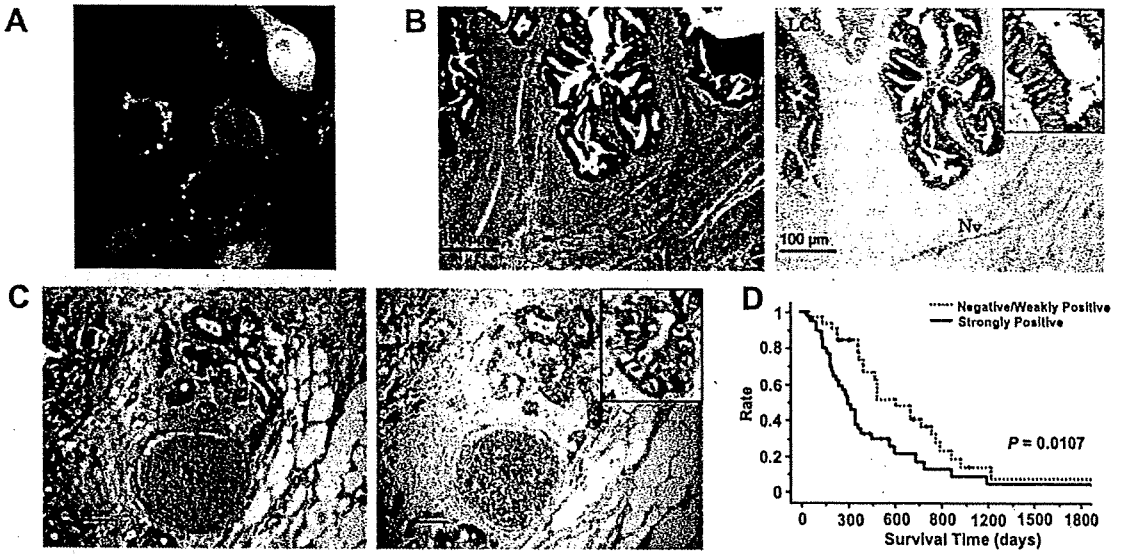

Fig. 3. Autophagosome formation in the surgically-resected specimens. (A) Autophagosome-incorporated LC3 protein was detected using GFP-LC3 fusion protein in a cultured colorectal cancer cell line, SW480, during amino acid-deprivation. (B) Tumor cell-specific LC3 accumulation in colorectal cancer tissue was detected using immunohistochemistry with anti-LC3 antibody and hematoxylin and eosin staining. (C) LC3 accumulation at the peripheral area of the pancreatic cancer tissue. Nv: nerve cells in which LC3 is constitutively expressed. (inlet) Higher magnification of (B) and (C). LC3 proteins were localized in the cytoplasm with irregular condensation. (D) The group with strongly positive expression of LC3 protein in the peripheral area of the pancreatic cancer tissue had a significantly shorter survival time. (A) and (B) are quoted from Ref. [26], (C) and (D) are quoted from Ref. [55].

conditions of clinical tumors. Furthermore, such models do not provide information regarding the initial steps of tumorigenesis, in which autophagy might have critical roles for pampering aberrantly proliferating cancer cells without extra blood supply. Examining the effect of various autophagy inhibitors in tumorigenic animals is another way to evaluate the roles of autophagy *in vivo*. But it has the drawback that these reagents are not completely autophagy-specific [32]. Recently, several genetically engineered mouse models targeting autophagy-related genes have been reported [12]. However, these mice often show embryonic or neonatal lethality, hampering the long-term observation of tumorigenesis in adult animals. Tissue-specific or inducible conditional knockout systems may be useful for this purpose.

Whether the control of autophagy is useful for cancer therapy and prevention is another important issue. Several small molecules are known to activate or inhibit autophagy (Fig. 1) [56]. Inhibiting the class I PI3K-mTOR axis by rapamycin induces autophagy. The inhibition of autophagy can be achieved by targeting several points. A class III PI3K inhibitor (3-methyladenine), lysosomotropic alkalines (chloroquine and 3-hydroxychloroquine), a lysosomal proton pump inhibitor (bafilomycin A1) and lysosomal enzyme inhibitors (E64d and pepstatin A) are available for experimental use. Since the contribution of autophagy to tumorigenesis is still controversial, we should carefully consider the application of autophagy inhibitors or activators for therapeutic use. Increasing evidence emphasizes the importance of basal autophagy for cellular quality control [57]. The disturbance of autophagy might result in unexpected adverse effects, especially in postmitotic cells in the central nervous system and cardiovascular system. The specificity of such activators and inhibitors is another issue to consider. The above-mentioned chemicals are not autophagy-specific. Also, the targeted kinases (PI3K, mTOR) or organelles (lysosome) have broad functions other than autophagy regulation.

In the last decade, autophagy has appeared in the center stage of cancer biology and is now attracting much attention in the development of new cancer therapeutics. As mentioned above, a considerable number of issues remain to be clarified. However, similar to its molecular process, the research field of autophagy is very dynamic. Novel strategies for monitoring autophagy and experimental models are appearing, and various small molecules regulating autophagic process will be available. Further investigation will reveal the dynamism of metabolism in cancer cells and tissues that is still behind the scene now. And it will likely open up a new field of cancer biology.

Conflicts of interest statement

None declared.

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References

[1] D.J. Klionsky, The molecular machinery of autophagy: unanswered questions, J. Cell Sci. 118 (2005) 7–18.

[2] D.J. Klionsky, Autophagy: from phenomenology to molecular understanding in less than a decade, Nat. Rev. Mol. Cell Biol. 8 (2007) 931–937.
[3] B. Levine, D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy, Dev. Cell 6 (2004) 463–477.
[4] N. Mizushima, Autophagy: process and function, Genes Dev. 21 (2007) 2861–2873.
[5] N. Mizushima, D.J. Klionsky, Protein turnover via autophagy: implications for metabolism, Annu. Rev. Nutr. 27 (2007) 19–40.
[6] D.J. Klionsky, J.M. Cregg, W.A. Dunn Jr., S.D. Emr, Y. Sakai, I.V. Sandoval, A. Sibirny, S. Subramani, M. Thumm, M. Veenhuis, Y. Ohsumi, A unified nomenclature for yeast autophagy-related genes, Dev. Cell 5 (2003) 539–545.
[7] P. Codogno, A.J. Meijer, Autophagy and signaling: their role in cell survival and cell death, Cell Death Differ. 12 (Suppl. 2) (2005) 1509–1518.
[8] Z. Xie, D.J. Klionsky, Autophagosome formation: core machinery and adaptations, Nat. Cell Biol. 9 (2007) 1102–1109.
[9] L. Yu, L. Strandberg, M.J. Lenardo, The selectivity of autophagy and its role in cell death and survival, Autophagy 4 (2008).
[10] M. Komatsu, T. Ueno, S. Waguri, Y. Uchiyama, E. Kominami, K. Tanaka, Constitutive autophagy: vital role in clearance of unfavorable proteins in neurons, Cell Death Differ. 14 (2007) 887–894.
[11] J.J. Lum, D.E. Bauer, M. Kong, M.H. Harris, C. Li, T. Lindsten, C.B. Thompson, Growth factor regulation of autophagy and cell survival in the absence of apoptosis, Cell 120 (2005) 237–248.
[12] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, Cell 132 (2008) 27–42.
[13] N. Mizushima, B. Levine, A.M. Cuervo, D.J. Klionsky, Autophagy fights disease through cellular self-digestion, Nature 451 (2008) 1069–1075.
[14] A.L. Harris, Hypoxia – a key regulatory factor in tumour growth, Nat. Rev. Cancer 2 (2002) 38–47.
[15] R.K. Jain, Molecular regulation of vessel maturation, Nat. Med. 9 (2003) 685–693.
[16] P. Vaupel, O. Thews, M. Hoeckel, Treatment resistance of solid tumors: role of hypoxia and anemia, Med. Oncol. 18 (2001) 243–259.
[17] M. Kitano, M. Kudo, K. Maekawa, Y. Suetomi, H. Sakamoto, N. Fukuta, R. Nakaoka, T. Kawasaki, Dynamic imaging of pancreatic diseases by contrast enhanced coded phase inversion harmonic ultrasonography, Gut 53 (2004) 854–859.
[18] K. Izuishi, K. Kato, T. Ogura, T. Kinoshita, H. Esumi, Remarkable tolerance of tumor cells to nutrient deprivation: possible new biochemical target for cancer therapy, Cancer Res. 60 (2000) 6201–6207.
[19] D.A. Nelson, T.T. Tan, A.B. Rabson, D. Anderson, K. Degenhardt, E. White, Hypoxia and defective apoptosis drive genomic instability and tumorigenesis, Genes Dev. 18 (2004) 2095–2107.
[20] G.L. Semenza, Mitochondrial autophagy: life and breath of the cell, Autophagy 4 (2008).
[21] S. Friberg, S. Mattson, On the growth rates of human malignant tumors: implications for medical decision making, J. Surg. Oncol. 65 (1997) 284–297.
[22] D.A. Rew, G.D. Wilson, Cell production rates in human tissues and tumours and their significance. Part II: clinical data, Eur. J. Surg. Oncol. 26 (2000) 405–417.
[23] K. Heidenreich, J.A. Hill, M. Hoyer-Hansen, X. Hu, W.P. Huang, A. Iwasaki, M. Jaattela, W.T. Jackson, X. Jiang, S. Jin, T. Johansen, J.U. Jung, M. Kadowaki, C. Kang, A. Kelekar, D.H. Kessel, J.A. Kiel, H.P. Kim, A. Kimchi, T.J. Kinsella, K. Kiselyov, K. Kitamoto, E. Knecht, et al, Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes, Autophagy 4 (2008) 151–175.
[24] G. Rez, S. Toth, Z. Palfia, Cellular autophagic capacity is highly increased in azaserine-induced premalignant atypical acinar nodule cells, Carcinogenesis 20 (1999) 1893–1898.
[25] E. Ogier-Denis, J.J. Houri, C. Bauvy, P. Codogno, Guanine nucleotide exchange on heterotrimeric Gi3 protein controls autophagic sequestration in HT-29 cells, J. Biol. Chem. 271 (1996) 28593–28600.
[26] K. Sato, K. Tsuchihara, S. Fujii, M. Sugiyama, T. Goya, Y. Atomi, T. Ueno, A. Ochiai, H. Esumi, Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation, Cancer Res. 67 (2007) 9677–9684.
[27] S.H. Lee, E.G. Jeong, N.J. Yoo, Mutational and expressional analysis of BNIP3, a pro-apoptotic Bcl-2 member, in gastric carcinomas, APMIS 115 (2007) 1274–1280.
[28] K. Degenhardt, R. Mathew, B. Beaudoin, K. Bray, D. Anderson, G. Chen, C. Mukherjee, Y. Shi, C. Gelinas, Y. Fan, D.A. Nelson, S. Jin, E. White, Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis, Cancer Cell 10 (2006) 51–64.
[29] V. Karantza-Wadsworth, S. Patel, O. Kravchuk, G. Chen, R. Mathew, S. Jin, E. White, Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis, Genes Dev. 21 (2007) 1621–1635.
[30] R. Mathew, S. Kongara, B. Beaudoin, C.M. Karp, K. Bray, K. Degenhardt, G. Chen, S. Jin, E. White, Autophagy suppresses tumor progression by limiting chromosomal instability, Genes Dev. 21 (2007) 1367–1381.
[31] M.A. Qadir, B. Kwok, W.H. Dragowska, K.H. To, D. Le, M.B. Bally, S.M. Gorski, Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization, Breast Cancer Res. Treat. (2008).
[32] R.K. Amaravadi, D. Yu, J.J. Lum, T. Bui, M.A. Christophorou, G.I. Evan, A. Thomas-Tikhonenko, C.B. Thompson, Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma, J. Clin. Invest. 117 (2007) 326–336.
[33] E. Tasdemir, M.C. Maiuri, L. Galluzzi, I. Vitale, M. Djavaheri-Mergny, M. D'Amelio, A. Criollo, E. Morselli, C. Zhu, F. Harper, U. Nannmark, C. Samara, P. Pinton, J.M. Vicencio, R. Carnuccio, U.M. Moll, F. Madeo, P. Paterlini-Brechot, R. Rizzuto, G. Szabadkai, G. Pierron, K. Blomgren, N. Tavernarakis, P. Codogno, F. Cecconi, G. Kroemer, Regulation of autophagy by cytoplasmic p53, Nat. Cell Biol. 10 (2008) 676–687.
[34] N. Furuya, J. Yu, M. Byfield, S. Pattingre, B. Levine, The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function, Autophagy 1 (2005) 46–52.
[35] X. Zeng, J.H. Overmeyer, W.A. Maltese, Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking, J. Cell Sci. 119 (2006) 259–270.
[36] X.H. Liang, S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh, B. Levine, Induction of autophagy and inhibition of tumorigenesis by beclin 1, Nature 402 (1999) 672–676.
[37] V.M. Aita, X.H. Liang, V.V. Murty, D.L. Pincus, W. Yu, E. Cayanis, S. Kalachikov, T.C. Gilliam, B. Levine, Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21, Genomics 59 (1999) 59–65.
[38] X. Qu, J. Yu, G. Bhagat, N. Furuya, H. Hibshoosh, A. Troxel, J. Rosen, E.L. Eskelinen, N. Mizushima, Y. Ohsumi, G. Cattoretti, B. Levine, Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene, J. Clin. Invest. 112 (2003) 1809–18020.
[39] Z. Yue, S. Jin, C. Yang, A.J. Levine, N. Heintz, Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor, Proc. Natl. Acad. Sci. USA 100 (2003) 15077–15082.
[40] C. Liang, P. Feng, B. Ku, I. Dotan, D. Canaani, B.H. Oh, J.U. Jung, Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG, Nat. Cell Biol. 8 (2006) 688–699.
[41] C. Liang, J.S. Lee, K.S. Inn, M.U. Gack, Q. Li, E.A. Roberts, I. Vergne, V. Deretic, P. Feng, C. Akazawa, J.U. Jung, Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking, Nat. Cell Biol. 10 (2008) 776–787.
[42] Y. Takahashi, D. Coppola, N. Matsushita, H.D. Cualing, M. Sun, Y. Sato, C. Liang, J.U. Jung, J.Q. Cheng, J.J. Mul, W.J. Pledger, H.G. Wang, Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis, Nat. Cell Biol. 9 (2007) 1142–1151.
[43] B. Ravikumar, C. Vacher, Z. Berger, J.E. Davies, S. Luo, L.G. Oroz, F. Scaravilli, D.F. Easton, R. Duden, C.J. O'Kane, D.C. Rubinsztein, Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease, Nat. Genet. 36 (2004) 585–595.
[44] S. Arico, A. Petiot, C. Bauvy, P.F. Dubbelhuis, A.J. Meijer, P. Codogno, E. Ogier-Denis, The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway, J. Biol. Chem. 276 (2001) 35243–35246.
[45] Z. Feng, H. Zhang, A.J. Levine, S. Jin, The coordinate regulation of the p53 and mTOR pathways in cells, Proc. Natl. Acad. Sci. USA 102 (2005) 8204–8209.
[46] H. Takeuchi, Y. Kondo, K. Fujiwara, T. Kanzawa, H. Aoki, G.B. Mills, S. Kondo, Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors, Cancer Res. 65 (2005) 3336–3346.
[47] D. Crighton, S. Wilkinson, J. O'Prey, N. Syed, P. Smith, P.R. Harrison, M. Gasco, O. Garrone, T. Crook, K.M. Ryan, DRAM, a p53-induced modulator of autophagy, is critical for apoptosis, Cell 126 (2006) 121–134.

[48] B. Inbal, S. Bialik, I. Sabanay, G. Shani, A. Kimchi, DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death, J. Cell Biol. 157 (2002) 455–468.
[49] P.G. Clarke, Developmental cell death: morphological diversity and multiple mechanisms, Anat. Embryol. Berl. 181 (1990) 195–213.
[50] Y. Kondo, T. Kanzawa, R. Sawaya, S. Kondo, The role of autophagy in cancer development and response to therapy, Nat. Rev. Cancer 5 (2005) 726–734.
[51] M.C. Maiuri, E. Zalckvar, A. Kimchi, G. Kroemer, Self-eating and self-killing: crosstalk between autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 8 (2007) 741–752.
[52] A. Mantovani, T. Schioppa, C. Porta, P. Allavena, A. Sica, Role of tumor-associated macrophages in tumor progression and invasion, Cancer Metastasis Rev. 25 (2006) 315–322.
[53] H. Aoki, Y. Kondo, K. Aldape, A. Yamamoto, E. Iwado, T. Yokoyama, E.F. Hollingsworth, R. Kobayashi, K. Hess, N. Shinojima, T. Shingu, Y. Tamada, L. Zhang, C. Conrad, O. Bogler, G. Mills, R. Sawaya, S. Kondo, Monitoring autophagy in glioblastoma with antibody against isoform b of human microtubule-associated protein 1 light chain 3, Autophagy 4 (2008).
[54] S. Jager, C. Bucci, I. Tanida, T. Ueno, E. Kominami, P. Saftig, E.L. Eskelinen, Role for Rab7 in maturation of late autophagic vacuoles, J. Cell Sci. 117 (2004) 4837–4848.
[55] S. Fujii, S. Mitsunaga, M. Yamazaki, T. Hasebe, G. Ishii, M. Kojima, T. Kinoshita, T. Ueno, H. Esumi, A. Ochiai, Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome, Cancer Sci. 99 (2008) 1813–1819.
[56] D.C. Rubinsztein, J.E. Gestwicki, L.O. Murphy, D.J. Klionsky, Potential therapeutic applications of autophagy, Nat. Rev. Drug Discov. 6 (2007) 304–312.
[57] T. Yorimitsu, D.J. Klionsky, Eating the endoplasmic reticulum: quality control by autophagy, Trends Cell Biol. 17 (2007) 279–285.

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Mitochondrial inhibitors show preferential cytotoxicity to human pancreatic cancer PANC-1 cells under glucose-deprived conditions

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ABSTRACT

Large areas of tumor are nutrient-starved and hypoxic due to a disorganized vascular system. Therefore, we screened small molecules to identify cytotoxic agents that function preferentially in nutrient-starved conditions. We found that efrapeptin F had preferential cytotoxicity to nutrient-deprived cells compared with nutrient-sufficient cells. Because efrapeptin F acts as a mitochondrial complex V inhibitor, we examined whether inhibitors of complex I, II, III, and V function as cytotoxic agents preferentially in nutrient-deprived cells. Interestingly, these inhibitors showed preferential cytotoxicity to nutrient-deprived cells and caused cell death under glucose-limiting conditions, irrespective of the presence or absence of amino acids and/or serum. In addition, these inhibitors were preferentially cytotoxic to nutrient-deprived cells even under hypoxic conditions. Further, efrapeptin F showed antitumor activity *in vivo*. These data indicate that mitochondrial inhibitors show preferential cytotoxicity to cancer cells under glucose-limiting conditions, and these inhibitors offer a promising strategy for anticancer therapeutic.

Introduction

Solid tumors have large areas starved for nutrients and oxygen that arise from immature and irregular distribution of blood vessels [1,2]. In particular, hypovascular tumors such as pancreatic cancers show an inherent ability to tolerate such severe growth conditions. Certain human pancreatic cancer cell lines, including PANC-1, AsPC-1, BxPC-3 and KP-3, exhibit marked environmental tolerance and can survive for prolonged periods of time in nutrient-deprived conditions [3]. Tolerance of these cancer cells to nutrient starvation has been associated with the activity of protein kinase B (PKB)/Akt. The PI3K-AKT-TOR signaling promotes cell proliferation and inhibits apoptosis. In addition, activation of Akt has been reported to stimulate cell survival, transformation, metastasis and angiogenesis [4,5]. Kigamicin D, a novel compound discovered from the culture broth of *Amycolatopsis* sp. ML630-mF1, blocks activation of Akt and exhibits preferential cytotoxicity to cancer cells under nutrient-deprived conditions compared to nutrient-rich conditions [6–8]. AG1024 and I-OMe-AG538, specific inhibitors of insulin-like growth factor-1 receptor tyrosine kinase, are also found to be cytotoxic to nutrient-deprived cells [9]. Therefore, agents active in nutrient-deprived conditions could function as anticancer agents.

Energy production is important for cell survival. The metabolism within a solid tumor is markedly different from that of the surrounding normal tissue [10–13]. Increased aerobic glycolysis is uniquely observed in cancers, thereby cancer cells use elevated amounts of glucose as a carbon source for anabolic reactions. However, part of the tumor is in a state of nutrient depletion. Tumor cells respond to nutrient-deprived conditions and adapt their metabolism to obtain amino acids. Autophagy is a catabolic process by which cells supply amino acids from self-digested organelles; cancer cells are likely to use autophagy to obtain amino acids as alternative energy sources [14]. Thus, their metabolic shift to the tumor microenvironment could represent a possible target for antitumor therapy. In this study, we screened natural products such as microbial metabolites to identify agents that preferentially reduce the survival of nutrient-deprived cancer cells. The screen identified efrapeptin F, which is produced by fungi and functions as a cytotoxic agent preferentially against human pancreatic cancer cells in glucose-limiting conditions.

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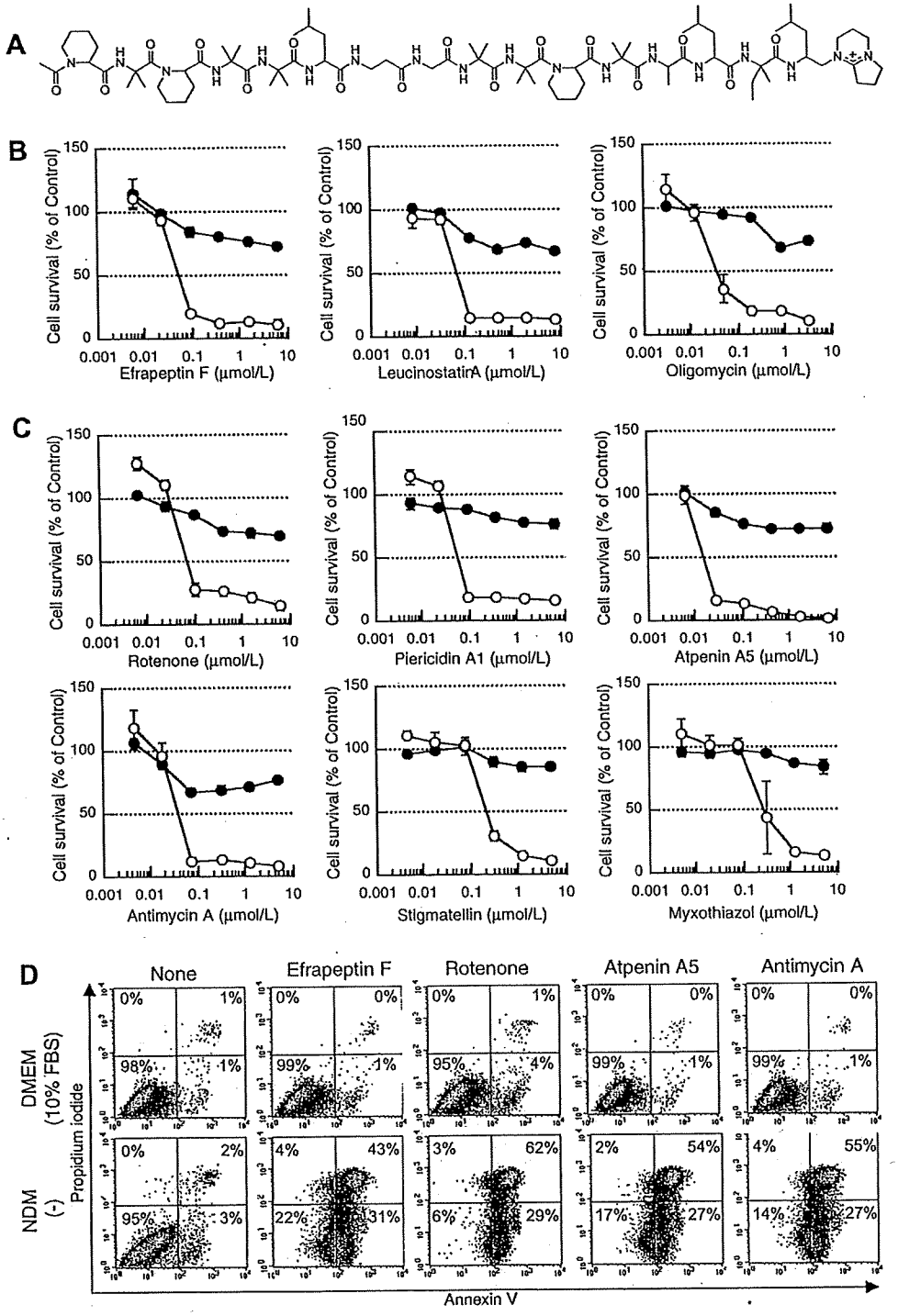
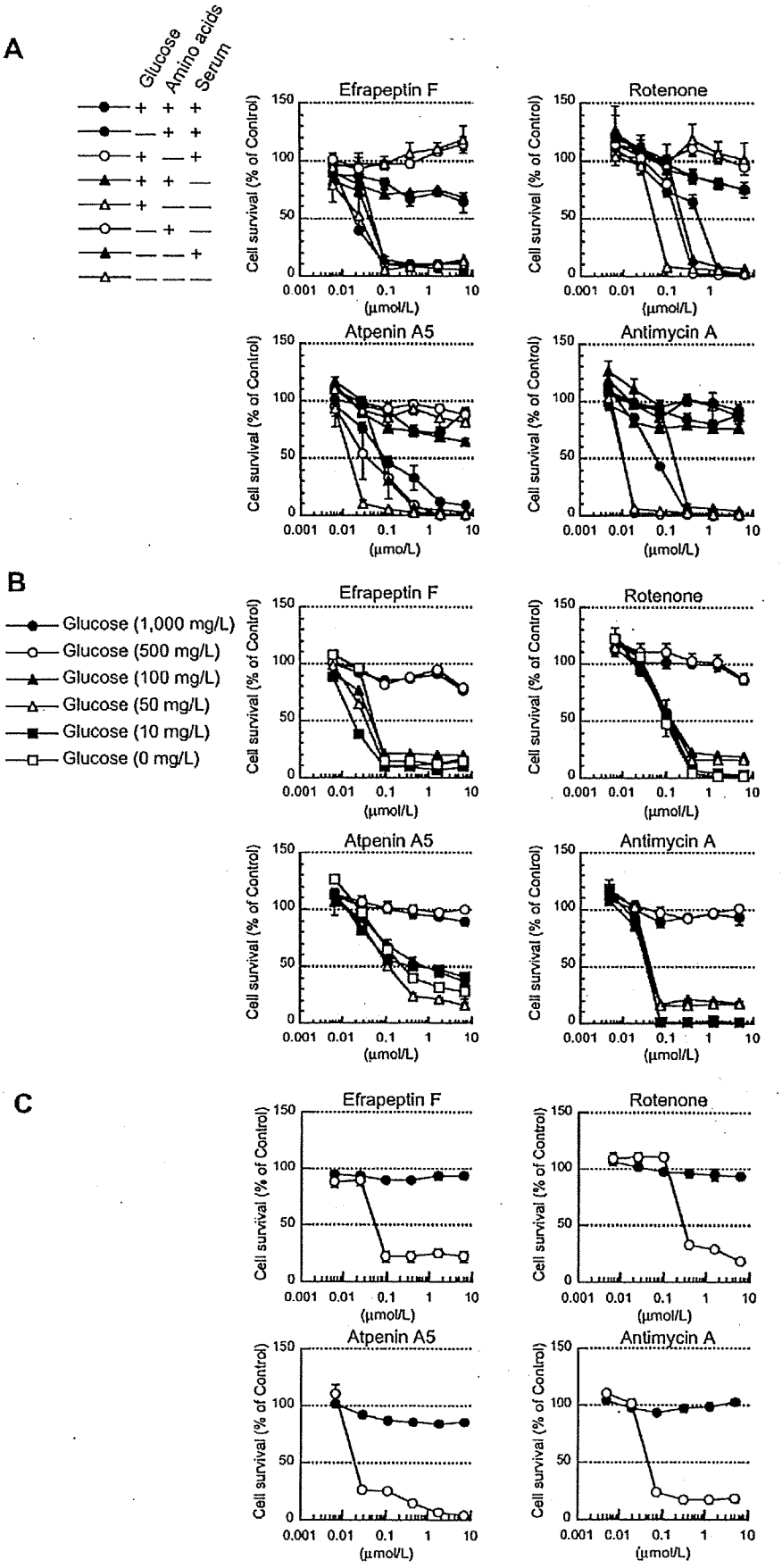


Fig. 1. Effect of efrapeptin F and mitochondrial inhibitors on PANC-1 survival under nutrient-deprived conditions. (A) Structure of efrapeptin F. (B) Effect of efrapeptin F and complex V inhibitors (leucinostatin A and oligomycin) on survival of PANC-1 cells in normal medium, DMEM (10% FBS) (●) and nutrient-deprived medium, NDM (−) (○). PANC-1 cells were incubated in DMEM (10% FBS) for 24 h. The cells were then washed with PBS and the medium was replaced with either fresh DMEM (10% FBS) or NDM (−). The indicated concentrations of efrapeptin F and complex V inhibitors were added to each well and the cells were incubated for 24 h. Cell viability was determined using the MTT assay. (C) Effect of complex I, II and III inhibitors on survival of PANC-1 cells in DMEM (10% FBS) (●) and NDM (−) (○). Rotenone and Piericidin A_1 were used as complex I inhibitors. Atpenin A_5 was as complex II inhibitors. Antimycin A, myxothiazol and stigmatellin were as complex III inhibitors. PANC-1 cells were incubated with inhibitors in DMEM (10% FBS) or NDM (−) for 24 h. (D) Flow cytometric analysis of PANC-1 cells treated with each inhibitor. PANC-1 cells were incubated with 0.1 μmol/L of mitochondrial inhibitors in DMEM (10% FBS) or NDM (−) for 24 h. The cells were stained with annexin V-FITC and propidium iodide and then analyzed using a flow cytometer.


A
Glucose
Amino acids
Serum
Efrapeptin F
Rotenone
Atpenin A5
Antimycin A
Cell survival (% of Control)
(μmol/L)
B
Glucose (1,000 mg/L)
Glucose (500 mg/L)
Glucose (100 mg/L)
Glucose (50 mg/L)
Glucose (10 mg/L)
Glucose (0 mg/L)
C

Materials and methods

Inhibitors. Efrapeptin F and Atpenin A_5 were purified from microbial culture extracts supplied by Meiji Seika Kaisha in our laboratory [15–18]. Rotenone and antimycin A were obtained from Sigma–Aldrich (St. Louis, MO).

Cell lines and culture conditions. Human pancreatic cancer PANC-1 cells and prostate cancer PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA), 100,000 U/L penicillin G, and 100 mg/L streptomycin. Nutrient starvation was achieved by culturing cells in nutrient-deprived medium (NDM) as previously described [9]. Briefly, the NDM composition was 265 mg/L $CaCl_2{\cdot}H_2O$, 400 mg/L KCl, 200 mg/L $MgSO_4{\cdot}7H_2O$, 6400 mg/L NaCl, 163 mg/L $NaH_2PO_4{\cdot}2H_2O$, 0.1 mg/L $Fe(NO_3)_3{\cdot}9H_2O$, 5 mg/L phenol red, 100,000 U/L penicillin G, 100 mg/L streptomycin, 25 mmol/L HEPES buffer (pH 7.4), and MEM vitamin solution (Invitrogen, Carlsbad, CA); the final pH was adjusted to 7.4 with 10% $NaHCO_3$.

Preferential cytotoxicity in nutrient-deprived conditions. PANC-1 cells (2.5×10^4 cells/well) in 96-well plates were cultured in DMEM (10% FBS) for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM (10% FBS) or NDM (−). Test samples were added to the well and cells were cultured for 24 h. Furthermore, the medium was replaced with DMEM (10% FBS) containing 0.5 mg/mL thiazolyl blue tetrazolium bromide (MTT; Sigma–Aldrich) and incubated for 3 h to determine cytotoxicity using the MTT assay [19]. Hypoxia was achieved by culturing cells with a mixture of 1% O_2, 5% CO_2 and 94% N_2.

Measurement of cellular ATP content. PANC-1 cells (2.5×10^4 cells/well) in 96-well plates were cultured in DMEM (10% FBS) for 24 h. The cells were washed with PBS and cultured in fresh DMEM (10% FBS) or NDM (−) with 0.25 μmol/L rotenone, 0.27 μmol/L atpenin A_5, 0.10 μmol/L antimycin A or 0.06 μmol/L efrapeptin F for 24 h. The ATP level in cells was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI).

Flow cytometric analysis. PANC-1 cells (5×10^5) in 60-mm dishes were incubated in DMEM (10% FBS) for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM (10% FBS) or NDM (−). Mitochondrial inhibitors (0.1 μmol/L) were added to the well and the cells were cultured for 24 h. The cells were incubated with annexin V-FITC and propidium iodide according to an annexin V-FITC apoptosis detection kit (Biovision Research Products, Mountain View, CA) and analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Animal experiments. Male severe combined immunodeficient (SCID) mice, 6 weeks old, were purchased from Charles River Japan (Yokohama, Japan) and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. PC-3 cells (1×10^7) were subcutaneously injected into the SCID mouse in the left lateral flank. Five days after inoculation, mice were divided randomly into test groups (control $n = 9$, efrapeptin F-treated $n = 7$) and efrapeptin F was intravenously administered twice weekly for 3 weeks to the efrapeptin F-treated group. Cisplatin was intravenously administered once weekly for 3 weeks. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × width^2)/2.

Statistical analysis. All data are representative of three independent experiments with similar results. The statistical data are expressed as mean ± SD using descriptive statistics. Statistical analysis was done by using Student's *t*-test.

Results

Efrapeptin F is preferentially cytotoxic to cancer cells in nutrient-deprived conditions

To identify cytotoxic agents that function preferentially on nutrient-deprived cancer cells, we screened the cultured media from various microorganisms. One extract of microbial cultured media exhibited preferential cytotoxicity to PANC-1 cells in nutrient-deprived medium (NDM (−)). The extract was subjected to chromatography to obtain a pure compound. The NMR and MS spectra data revealed its chemical structure to be efrapeptin F (Fig. 1A) [15,16]. Efrapeptin F exhibited preferential cytotoxicity to PANC-1 cells in NDM (−), but not in nutrient-sufficient medium (DMEM (10% FBS)) (Fig. 1B). The cytotoxic effect of efrapeptin F on PANC-1 cells in NDM (−) (IC_{50} = 0.052 μmol/L) was more than 100 times stronger than in DMEM (10% FBS) (IC_{50} = >10 μmol/L).

Mitochondrial inhibitors are preferentially cytotoxic to cancer cells in nutrient-deprived conditions

Efrapeptin F has been previously reported to act as an inhibitor of mitochondrial F_1F_0-ATPase (complex V) [17]. Therefore, we examined whether mitochondrial complex V inhibitors function as cytotoxic agents preferentially on nutrient-deprived cells (Fig. 1B). Interestingly, leucinostatin A and oligomycin (complex V inhibitors) were more cytotoxic to PANC-1 cells in NDM (−) compared with DMEM (10% FBS) [20,21]. In addition, rotenone and piericidin A_1 (NADH-ubiquinone reductase (complex I) inhibitors), atpenin A_5 (a succinate-ubiquinone reductase (complex II) inhibitor), antimycin A, stigmatellin and myxothiazol (ubiquinone-cytochrome *c* (complex III) inhibitors) also were more cytotoxic to PANC-1 cells in NDM (−) compare to DMEM (10% FBS) (Fig. 1C) [20–22]. These results clearly demonstrate that mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived PANC-1 cells. Efrapeptin F (a complex V inhibitor), rotenone (a complex I inhibitor), atpenin A_5 (a complex II inhibitor), and antimycin A (a complex III inhibitor) were selected for further study. The mode of cell death caused by mitochondrial inhibitors in nutrient-deprived conditions was examined using annexin V-FITC and propidium iodide double staining and flow cytometry. Mitochondrial inhibitors significantly increased the early-apoptotic and late-apoptotic cells in nutrient-deprived conditions, but not to nutrient-sufficient conditions (Fig. 1D). These results suggested that these inhibitors induce apoptosis in nutrient-deprived cells.

Mitochondrial inhibitors are preferentially cytotoxic to cancer cells only under glucose-limiting conditions

To determine what nutrient component was responsible for cytotoxicity of mitochondrial inhibitors, we examined the effect

Fig. 2. Effect of mitochondrial inhibitors on PANC-1 survival under glucose-starved conditions and hypoxic conditions. (A) Effect of nutrient starvation on cytotoxicity of mitochondrial inhibitors. PANC-1 cells were incubated with inhibitors in nutrient-deprived medium containing glucose, amino acids and/or dialyzed FBS for 24 h. (B) Effect of glucose levels on cytotoxicity of mitochondrial inhibitors. PANC-1 cells were incubated with inhibitors in DMEM (10% dialyzed FBS) containing the indicated concentrations of glucose for 24 h. (C) Effect of hypoxia on cytotoxicity of mitochondrial inhibitors. PANC-1 cells were incubated with inhibitors in DMEM (10% FBS) (●) or NDM (−) (○) under 1% O_2 for 24 h.

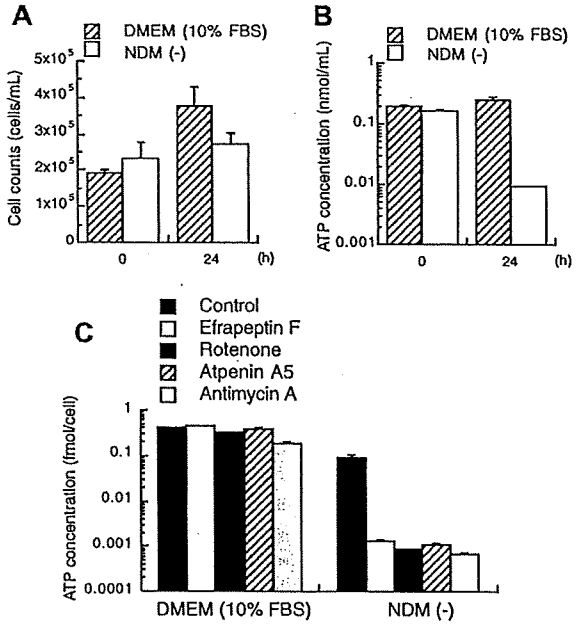


Fig. 3. Effect of mitochondrial inhibitors on cellular ATP levels of PANC-1 cells grown in nutrient-deprived medium. (A) Effect of nutrient starvation on PANC-1 cell growth. PANC-1 cells were incubated in DMEM (10% FBS) or NDM (−) for 24 h and cell numbers were measured by cell counting. (B) Cellular ATP levels were determined by the CellTiter-Glo Luminescent Cell Viability Assay after incubation in DMEM (10% FBS) or NDM (−) for 24 h. (C) PANC-1 cells were incubated with 0.25 μmol/L rotenone, 0.27 μmol/L atpenin A_5, 0.10 μmol/L antimycin A and 0.06 μmol/L efrapeptin F in DMEM (10% FBS) or NDM (−) for 24 h and cellular ATP levels were determined.

of these inhibitors on PANC-1 cell survival under various nutrient-starved conditions (Fig. 2A). Mitochondrial inhibitors preferentially induced cell death under glucose-deprived conditions, irrespective of the presence or absence of amino acids and/or serum. We then examined the effect of glucose levels on cytotoxicity of these inhibitors (Fig. 2B). The concentration of glucose in DMEM is 1000 mg/L. Mitochondrial inhibitors did not induce cell death in the PANC-1 cells cultured with 1000 and 500 mg/L glucose, but in less than 100 mg/L glucose each inhibitor exhibited cytotoxicity. These results demonstrate clearly that glucose is the key component to determine the sensitivity of cancer cells to mitochondrial inhibitors.

Mitochondrial inhibitors are preferentially cytotoxic to nutrient-deprived cells under hypoxic conditions

Because large areas of tumor are exposed not only to nutrient starvation but also to hypoxic conditions, we examined preferential cytotoxicity of mitochondrial inhibitors to nutrient-deprived cells in hypoxic conditions (Fig. 2C). These inhibitors were more cytotoxic to nutrient-deprived PANC-1 cells in 1% O_2 as well as 21% O_2. Our results demonstrate that mitochondrial inhibitors show preferentially cytotoxicity to nutrient-deprived cells not only under normoxic conditions but also under hypoxic conditions.

Reduction of cellular ATP levels by mitochondrial inhibitors induces preferential cell death to nutrient-deprived cells

To investigate why mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived cells, we examined the effect of mitochondrial inhibitors on cellular ATP levels in nutrient-deprived cells. When PANC-1 cells were incubated in NDM (−) for 24 h, the cells grew less and the cellular ATP levels were markedly decreased (Fig. 3A and B). Since PANC-1 cells incubated in NDM (−) for 24 h could hardly be stained by trypan blue, the cells were able to survive in nutrient starvation in spite of lower ATP levels (Fig. S1). When PANC-1 cells were exposed to mitochondrial inhibitors for 24 h, the amount of cellular ATP were slightly decreased in DMEM (10% FBS), whereas in NDM (−) cellular ATP content decreased 100-fold compared to controls (Fig. 3C). These results indicate that depletion of ATP exerts preferential cytotoxicity to nutrient-starved cells.

Table 1
Growth inhibitory activity of efrapeptin F against 39 human cancer cell lines in the JFCR39 panel.

Origin of cancer	Cell line	Log GI_{50} (μmol/L)[a]
Breast	HBC-4	−7.22
	BSY-1	−6.73
	HBC-5	−8.00
	MCF-7	−8.00
	MDA-MB-231	−5.94
Central nervous system	U251	−7.45
	SF-268	−6.17
	SF-295	−8.00
	SF-539	−6.13
	SNB-75	−5.79
	SNB-78	−6.47
Colon	HCC2998	−6.84
	KM-12	−6.65
	HT-29	−6.86
	HCT-15	−5.61
	HCT-116	−6.48
Lung	NCI-H23	−8.00
	NCI-H226	−6.60
	NCI-H522	−8.00
	NCI-H460	−6.69
	A549	−6.53
	DMS273	−6.64
	DMS114	−8.00
Melanoma	LOX-IMVI	−6.71
Ovary	OVCAR-3	−6.58
	OVCAR-4	−5.85
	OVCAR-5	−6.21
	OVCAR-8	−8.00
	SK-OV-3	−6.46
Kidney	RXF-631L	−5.17
	ACHN	−5.94
Stomach	St-4	−6.10
	MKN1	−6.56
	MKN7	−8.00
	MKN28	−8.00
	MKN45	−6.78
	MKN74	−8.00
Prostate	DU-145	−6.76
	PC-3	−8.00
MG-MID[b]		−6.87
Delta[c]		1.13
Range[d]		2.83

[a] Log concentration of efrapeptin F for inhibition of cell growth at 50% compared to control.
[b] Mean value of log GI_{50} over all cell lines tested.
[c] The difference in log GI_{50} value of the most sensitive cell and MG-MID value.
[d] The difference in log GI_{50} value of the most sensitive cell and the least sensitive cell.

Efrapeptin F inhibits tumor growth in vivo

PANC-1 cells are low tumorigenicity even in immunodeficient mice. To explore the *in vivo* antitumor activity of mitochondrial inhibitors, we examined the growth inhibitory activity of efrapeptin F against 39 human cancer cell lines of the JFCR39 panel (Table 1) [23–25]. Efrapeptin F exhibited potent growth inhibitory

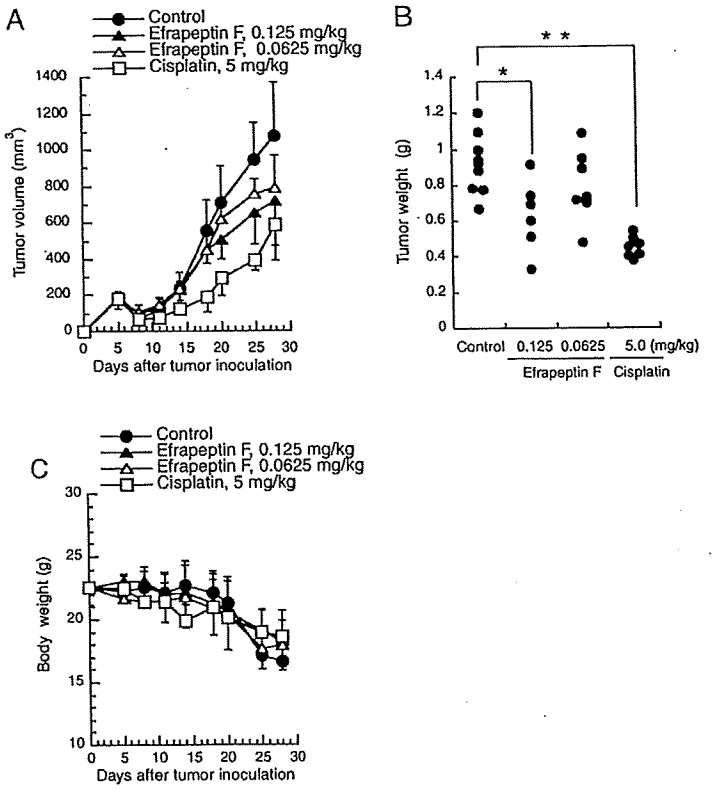


Fig. 4. Antitumor effect of efrapeptin F on PC-3 cells in SCID mice. PC-3 cells (1×10^7) were subcutaneously inoculated into SCID mice on day 0. Efrapeptin F was administrated intravenously twice weekly for 3 weeks from day 5. (A) Tumor volumes. *Y* axis, tumor volume (mm^3); *X* axis, time (day). (B) Tumor weight. The tumors were excised on day 28. $^{**}P < 0.001$; $^{*}P < 0.05$, compared with control (Student's *t*-test). (C) Body weight. *Y* axis, body weight (g); *X* axis, time (day). *Points*, mean values; *bars*, SD.

activity, and the mean value for log concentration for inhibition of cell growth at 50% compared to control was −6.87 (135 μmol/L). In particular, HBC-5, MCF-7, SF-295, NCI-H23, NCI-H522, DMS114, OVCAR-8, MKN7, MKN28, MKN74 and PC-3 cells were sensitive to efrapeptin F. Efrapeptin F showed preferential cytotoxicity to PC-3 cells in nutrient-deprived conditions as well as to PANC-1 cells (Fig. S2). Therefore, xenograft models of PC-3 cells were used to evaluate the *in vivo* antitumor activity of efrapeptin F. Efrapeptin F was intravenously administered twice weekly for 3 weeks from day 5 after the tumor inoculation. Efrapeptin F inhibited tumor growth of the PC-3 xenograft (Fig. 4A and B). Efrapeptin F at 0.125 and 0.0625 mg/kg reduced tumor weight by 68% and 86%, respectively (Tumor weight (g), control = 0.92 ± 0.17 (mean ± SD), 0.125 mg/kg efrapeptin F = 0.63 ± 0.20, 0.0625 mg/kg efrapeptin F = 0.79 ± 0.20) (Fig. 4B). To assess toxicity, we measured the body weight of the tumor-bearing mice (Fig. 4C). Their weight was not reduced by administration of efrapeptin F at these doses. However, among seven mice that were administrated efrapeptin F at a high dose (125 μg/kg), only one mouse died at day 23. Remaining mice survived until the end of the experiment without a decrease of body weight and anatomically without toxic effects in critical organs.

Discussion

Tumor microenvironment strongly affects tumor development and progression. Many aspects of physiology that differentiate solid tumors from normal tissues arise from differences in vasculature. Disorganized vascular systems in tumors result in large areas of tumor exposed to nutrient starvation and hypoxic conditions. In addition, due to the unregulated growth of tumor cells caused by genetic and epigenetic alterations, tumor cells proliferate more rapidly than normal cells and nutrient and oxygen demands often exceed supply [26–28]. In particular, highly aggressive tumor cells such as pancreatic cancers that are relatively hypovascular, are able to survive even in conditions of low nutrients and low oxygen supply. Since chronic nutrient deprivation seldom occurs in normal tissues, one strategy for anticancer agent development is to target cancer cells growing in nutrient-deprived conditions. Thus, we screened to identify cytotoxic agents that function preferentially in nutrient-deprived cancer cells.

Previous studies have shown that conventional chemotherapeutic drugs and various small molecule inhibitors were only weakly cytotoxic to cancer cells in nutrient-deprived conditions [9]. In this study, we found that the small molecule efrapeptin F, which is produced by some fungi showed preferential cytotoxicity to PANC-1 cells grown in nutrient-deprived conditions compared with cells in nutrient-sufficient conditions. Because efrapeptin F inhibits the mitochondrial complex V, we examined whether mitochondrial complex V inhibitors such as leucinostatin A and oligomycin act as cytotoxic agents preferentially on nutrient-deprived cells. Interestingly, these inhibitors were more cytotoxic to PANC-1 cells in NDM (−) compared to DMEM (10% FBS). In addition, mitochondrial complex I inhibitors (rotenone and piericidin A_1), a complex II inhibitor (atpenin A_5), and complex III inhibitors (antimycin A, stigmatellin and myxothiazol) also were more cytotoxic to PANC-1 cells in NDM (−). These results clearly demonstrate that mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived PANC-1 cells, suggesting that mitochondrial inhibitors have unique and attractive characteristics in antitumor agent development. These inhibitors induced cell death under glucose-limiting conditions, irrespective of the presence or absence of amino acids and/or serum. The glucose concentration in colon cancers is only ~1 of 45 of typical plasma glucose concentration (1000 mg/L

or 5.6 nmol/L) [13]. Mitochondrial inhibitors did not induce cell death in 1000 mg/L glucose, but each inhibitors exhibited cytotoxicity in less than 100 mg/L glucose levels. The cytotoxicity caused by mitochondrial inhibitors depended on glucose levels in the culture medium and glucose was the key component to determine the sensitivity of cancer cells to their inhibitors. However, it is unclear how mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived cells. The cellular ATP level was markedly decreased in PANC-1 cells grown in nutrient starvation. Mitochondrial inhibitors induced ATP depletion in nutrient-deprived cells at lower concentrations of inhibitors compared with nutrient-sufficient cells, thereby these inhibitors could exert preferential cytotoxicity under nutrient-deprived conditions.

Large areas of tumor are exposed not only to nutrient starvation but also hypoxic conditions. Therefore, we examined preferential cytotoxicity of mitochondrial inhibitors to nutrient-deprived cells in hypoxic conditions. Mitochondrial inhibitors showed preferential cytotoxicity to nutrient-deprived cells not only under hypoxic conditions but also under normoxic conditions. Normal tissue uses glycolysis to generate approximately 10% of the cellular ATP, with mitochondria accounting for 90%. In tumor sections, however, over 50% of the cellular ATP is produced by glycolysis with the remainder being generated at the mitochondria [29]. In hypoxic conditions (1% O_2), HIF-1α was stabilized and accumulated in nutrient-deprived PANC-1 cells, and the real-time PCR analysis revealed that hexokinase 2 and glucose transporter-1 expression were increased (data not shown). Despite PANC-1 cells grown in nutrient-deprived and hypoxic conditions were represented activation of glycolysis and induction of glucose transporter-1, mitochondrial inhibitors exhibited strong cytotoxicity to these cells. Therefore, ATP generation by mitochondria appeared to be essential for cell survival under hypoxic as well as normoxic conditions.

PANC-1 cells are low tumorigenicity even in SCID mice. To examine the *in vivo* antitumor activity of mitochondrial inhibitors, we explored cancer cell lines that were more sensitive to efrapeptin F. The growth inhibitory activity of efrapeptin F against 39 human cancer cell lines of the JFCR39 panel revealed that human prostate cancer PC-3 cells were highly sensitive to efrapeptin F. Thus, PC-3 cancer xenograft models were used to evaluate *in vivo* antitumor activity, and efrapeptin F was found to induce regression of PC-3 xenograft tumors. In this study, we demonstrated that mitochondrial inhibitors showed preferential cytotoxicity to nutrient-deprived cancer cells relative to nutrient-sufficient cells. Therefore, the potent cytotoxicity of these inhibitors to cancer cells deprived of nutrients (simulating a tumor microenvironment) makes mitochondria a promising target for new drugs that may be developed to treat a broad spectrum of malignant tumors.

Acknowledgments

This work was supported by a Grant-in-Aid for the Third-Term Comprehensive 10-Years Strategy for Cancer Control from the Ministry of Health, Labour and Welfare in Japan. We thank Ms. S. Kakuda for technical assistance and the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from the Ministry of Education, Culture, Sports, Science and Technology, Japan for supplying the measurement of growth inhibitory activities on 39 human cancer cell lines.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.050.

References

[1] P. Vaupel, F. Kallinowski, P. Okunieff, Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review, Cancer Res. 49 (1989) 6449–6465.
[2] J.M. Brown, A.J. Giaccia, The unique physiology of solid tumors: opportunities (and problems) for cancer therapy, Cancer Res. 58 (1998) 1408–1416.
[3] K. Izuishi, K. Kato, T. Ogura, T. Kinoshita, H. Esumi, Remarkable tolerance of tumor cells to nutrient deprivation: possible new biochemical target for cancer therapy, Cancer Res. 60 (2000) 6201–6207.
[4] R. Baserga, The contradictions of the insulin-like growth factor 1 receptor, Oncogene 19 (2000) 5574–5581.
[5] M.N. Pollak, E.S. Schernhammer, S.E. Hankinson, Insulin-like growth factors and neoplasia, Nat. Rev. Cancer 4 (2004) 505–518.
[6] S. Kunimoto, J. Lu, H. Esumi, Y. Yamazaki, N. Kinoshita, Y. Honma, M. Hamada, M. Ohsono, M. Ishizuka, T. Takeuchi, Kigamicins, novel antitumor antibiotics. I. Taxonomy, isolation, physico-chemical properties and biological activities, J. Antibiot. 56 (2003) 1004–1011.
[7] S. Kunimoto, T. Someno, Y. Yamazaki, J. Lu, H. Esumi, H. Naganawa, Kigamicins, novel antitumor antibiotics. II. Structure determination, J. Antibiot. 56 (2003) 1007–1012.
[8] J. Lu, S. Kunimoto, Y. Yamazaki, M. Kaminishi, H. Esumi, H. Esumi, Kigamicin D, a novel anticancer agent based on a new anti-austerity strategy targeting cancer cells' tolerance to nutrient starvation, Cancer Sci. 95 (2004) 547–552.
[9] I. Momose, S. Kunimoto, M. Osono, D. Ikeda, Inhibitors of insulin-like growth factor-1 receptor tyrosine kinase are preferentially cytotoxic to nutrient-deprived pancreatic cancer cells, Biochem. Biophys. Res. Commun. 380 (2009) 171–176.
[10] N.C. Denko, Hypoxia, HIF1 and glucose metabolism in the solid tumour, Nat. Rev. Cancer 8 (2008) 705–723.
[11] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis?, Nat Rev. Cancer 4 (2004) 891–899.
[12] G. Kroemer, J. Pouyssegur, Tumor cell metabolism: cancer's Achilles' heel, Cancer Cell 13 (2008) 472–482.
[13] A. Hirayama, K. Kami, M. Sugimoto, M. Sugawara, N. Toki, H. Onozuka, T. Kinoshita, N. Saito, A. Ochiai, M. Tomita, H. Esumi, T. Soga, Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry, Cancer Res. 69 (2009) 4918–4925.
[14] B. Levine, D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy, Dev. Cell 6 (2004) 463–477.
[15] S. Gupta, B.S. Krasnoff, W.D. Roberts, A.A.J. Renwick, S.L. Brinen, J. Clardy, Structures of the efrapeptins: potent inhibitors of mitochondrial ATPase from the fungus *Tolypocladium niveum*, J. Am. Chem. Soc. 113 (1991) 707–709.
[16] S. Gupta, B.S. Krasnoff, W.D. Roberts, A.A.J. Renwick, S.L. Brinen, J. Clardy, Structure of efrapeptins from the fungus *Tolypocladium niveum*: peptide inhibitors of mitochondrial ATPase, J. Org. Chem. 57 (1992) 2306–2313.
[17] R.L. Cross, W.E. Kohlbrenner, The mode of inhibition of oxidative phosphorylation by efrapeptin (A23871). Evidence for an alternating site mechanism for ATP synthesis, J. Biol. Chem. 253 (1978) 4865–4873.
[18] M. Kawada, I. Momose, T. Someno, G. Tsujiuchi, D. Ikeda, New atpenins, NBRI23477A and B, inhibit the growth of human prostate cancer cells, J. Antibiot. 62 (2009) 243–246.
[19] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[20] M. Ueki, K. Machida, M. Takeuchi, Antifungal inhibitors of mitochondrial respiration: discovery and prospects for development, Curr. Opin. Investig. Drugs 2 (2000) 387–398.
[21] N. Orme-Johnson, Direct and indirect inhibitors of mitochondrial ATP synthesis, Methods Cell Biol. 80 (2007) 813–826.
[22] H. Miyadera, K. Shiomi, H. Ui, Y. Yamaguchi, R. Masuma, H. Tomoda, H. Miyoshi, A. Osanai, K. Kita, S. Omura, Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate-ubiquinone oxidoreductase), Proc. Natl. Acad. Sci. USA 100 (2003) 473–477.
[23] S. Dan, T. Tsunoda, O. Kitahara, R. Yanagawa, H. Zembutsu, T. Katagiri, K. Yamazaki, Y. Nakamura, T. Yamori, An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines, Cancer Res. 62 (2002) 1139–1147.
[24] T. Yamori, Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics, Cancer Chemother. Pharmacol. 52 (Suppl. 1) (2003) S74–S79.
[25] T. Yamori, A. Matsunaga, S. Sato, K. Yamazaki, A. Komi, K. Ishizu, I. Mita, H. Edatsugi, Y. Matsuba, K. Takezawa, O. Nakanishi, H. Kohno, Y. Nakajima, H. Komatsu, T. Andoh, T. Tsuruo, Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an in vitro and in vivo human cancer cell line panel, Cancer Res. 59 (1999) 4042–4049.
[26] C.V. Dang, G.L. Semenza, Oncogenic alterations of metabolism, Trends Biochem. Sci. 24 (1999) 68–72.
[27] R.M. Southerland, Cell and environment interactions in tumor microregions: the multicell spheroid model, Science 240 (1988) 178–184.
[28] G. Helmlinger, F. Yuan, M. Dellian, R.K. Jain, Interstitial pH and pO_2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation, Nat. Med. 3 (1997) 177–182.
[29] O. Warburg, On respiratory impairment in cancer cells, Science 124 (1956) 269–270.

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Inhibitors of insulin-like growth factor-1 receptor tyrosine kinase are preferentially cytotoxic to nutrient-deprived pancreatic cancer cells

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ABSTRACT

Chronic deprivation of nutrients is rare in normal tissues, however large areas of tumor are nutrient-starved and hypoxic due to a disorganized vascular system. Some cancers show an inherent ability to tolerate severe growth conditions. Therefore, we screened chemical compounds to identify cytotoxic agents that function preferentially in nutrient-deprived conditions. We found that AG1024, a specific inhibitor of insulin-like growth factor-1 receptor tyrosine kinase (IGF-1R), showed preferential cytotoxicity to human pancreatic cancer cells in nutrient-deprived conditions relative to cells in nutrient-sufficient conditions. The cytotoxicity of I-OMe-AG538 (another specific inhibitor of IGF-1R kinase) was also enhanced in nutrient-deprived cells. In addition, AG1024 and I-OMe-AG538 potently inhibited IGF-1R activation to nutrient-deprived cells. In contrast, conventional chemotherapeutic drugs, as well as inhibitors of PDGFR and EGFR kinases, elicited weak cytotoxicity. These data indicate that nutrient-deprived human pancreatic cancer cells have increased sensitivity to inhibition of IGF-1R activation. IGF-1R inhibitors offer a promising strategy for anticancer therapeutic approaches that are oriented toward tumor microenvironment.

Patients diagnosed with pancreatic cancer, an aggressive disease with the lowest 5-year survival rates of all cancers, develop metastases rapidly and die within a short period of time after diagnosis [1,2]. Pancreatic cancer is largely resistant to almost all known chemotherapeutic agents, including 5-fluorouracil, paclitaxel and doxorubicin; surgery is the only current treatment modality that offers any prospect of potential cure. Clearly, there is a dire need for new therapeutic alternatives that improve clinical outcome for pancreatic cancer patients.

Tumor microenvironment exerts an important influence on cancer physiology. The disorganized vascular system in a tumor often results in large areas of tumor starved for nutrients and oxygen. Pancreatic cancers in particular, which are characterized as hypovascular tumors, show an inherent ability to tolerate severe growth conditions. Certain human pancreatic cancer cell lines, including PANC-1, AsPC-1, BxPC-3 and KP-3, exhibit marked environmental tolerance and can survive for prolonged periods of time in nutrient-deprived conditions. Because tolerance of these cancer cells to nutrient starvation has been associated with the activity of protein kinase B (PKB)/Akt [3], it has been hypothesized that agents that diminish such tolerance could function as anticancer agents [4–7].

Insulin-like growth factors-1 (IGF-1) and -2 (IGF-2) are involved in the pathophysiology of a wide range of human neoplasias due to the mitogenic and antiapoptotic properties mediated by their type 1 receptor (IGF-1R) [8]. IGF-1R is a tetrameric transmembrane receptor tyrosine kinase composed of two α and β subunits. The extracellular α subunit is responsible for ligand binding, whereas the β subunit consists of a transmembrane domain and an intracellular tyrosine kinase domain [9,10]. Ligand binding activates the intrinsic receptor tyrosine kinase, resulting in trans-β subunit autophosphorylation and stimulation of PI3K-AKT-TOR and RAF-MAPK signaling pathways. In addition to cell proliferation, activation of IGF-1R has been reported to stimulate cell survival, transformation, metastasis and angiogenesis [11]. Targeted inhibition of IGF-1R signaling has been shown to result in impressive anti-neoplastic activity in many *in vitro* and *in vivo* models of common human cancers. IGF-1R small interfering RNAs [12], anti-receptor antibodies [13,14], a IGF-1-like competitive peptide antagonist [15], a dominant-negative IGF-1R [16–18] and small-molecule IGF-1R tyrosine kinase inhibitors [19,20] have all been found to interfere with cell growth and proliferation. IGF-1R is therefore regarded as an attractive potential target in the development of new drugs to treat malignant tumors.

In this study, we screened chemical compounds to identify agents that preferentially reduce the survival of nutrient-deprived human pancreatic cancer PANC-1 cells. The screen identified IGF-1R inhibitors, which function as cytotoxic agents preferentially on human pancreatic cancer cells in nutrient-deprived conditions.

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Materials and methods

Materials. Antibodies used in Western blotting included anti-IGF-1Rβ (sc-713), anti-Erk 1 (sc-93) and anti-phospho-Erk (sc-7383) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-α-tubulin (T5168) from Sigma–Aldrich (St. Louis, MO); and anti-Akt (#9272), anti-phospho-Akt (Ser 473) (#9271), anti-phospho-Akt (Thr 308) (#9275) and anti-phospho-IGF-1R (#3021) from Cell Signaling Technology (Denvers, MA). Recombinant human IGF-1 was from R&D Systems (Minneapolis, MN). AG1024, AG1296, AG1478 and I-OMe-AG538 were obtained from Calbiochem (Madison, WI). Doxorubicin hydrochloride, fluorouracil, paclitaxel and mitomycin C were from Sigma. The SCADS inhibitor kit I consisting of 79 chemical inhibitors with ~60 different targets was kindly provided by the Screening Committee on Anticancer Drugs (Japan).

Cells and culture. Human pancreatic cancer cell lines PANC-1, Capan-1, MIA Paca-2, BxPC-3 and PSN-1 were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA), 100,000 U/L penicillin G, and 100 mg/L streptomycin. Nutrient starvation was achieved by culturing the cells in nutrient-deprived medium (NDM) as previously described [3–7]. Briefly, the composition of the NDM was as follows: 265 mg/L $CaCl_2{\cdot}H_2O$, 400 mg/L KCl, 200 mg/L $MgSO_4{\cdot}7H_2O$, 6400 mg/L NaCl, 163 mg/L $NaH_2PO_4{\cdot}2H_2O$, 0.1 mg/L $Fe(NO_3)_3{\cdot}9H_2O$, 5 mg/L phenol red, 100,000 U/L penicillin G, 100 mg/L streptomycin, 25 mmol/L HEPES buffer (pH 7.4), and MEM vitamin solution (Invitorogen, Carlsbad, CA); the final pH was adjusted to 7.4 with 10% $NaHCO_3$.

Preferential cytotoxicity in nutrient-deprived conditions. PANC-1 cells (2.5×10^4 cells/well) in 96-well plates were cultured in DMEM for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM or NDM. Test samples were added to the well and cells were cultured for 24 h. Cytotoxicity was determined using the MTT assay [21].

Preparation of cell lysate and Western blotting. PANC-1 cells (5×10^5) in 35-mm dishes were incubated in DMEM for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM or NDM. AG1024 or I-OMe-AG538 was added to each dish and the cells were incubated for 1 h prior to stimulation with 50 ng/ml IGF-1 for 10 min. The cells were washed twice with ice-cold PBS containing 100 μm Na_3VO_4 and then lysed in a lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM Na_3VO_4, pH 7.5, and 25 μg/ml each of antipain, leupeptin, and pepstatin). Equal amounts of protein extract were separated by SDS–polyacrylamide gel electrophoresis, transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with anti-IGF-1R, anti-phospho-IGF-1R, anti-Akt, anti-phospho-Akt (Thr 308), anti-phospho-Akt (Ser 473), anti-Erk 1,

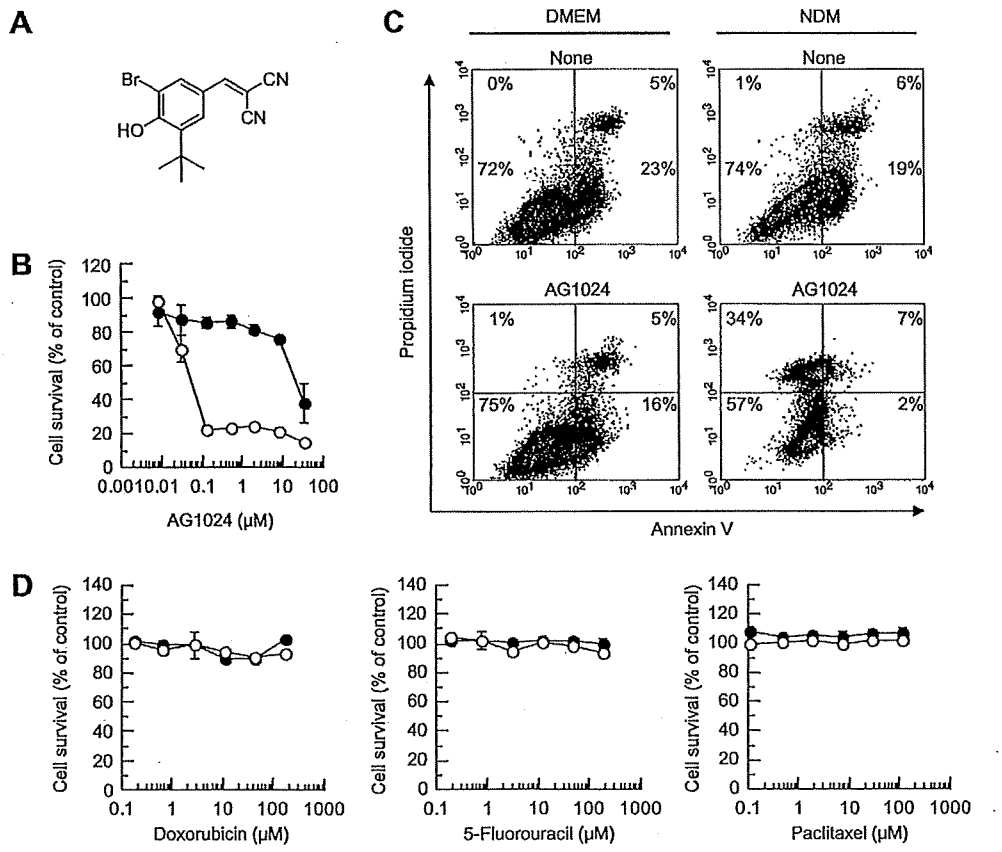

Fig. 1. Effect of AG1024 on survival of PANC-1 cells in nutrient-deprived conditions. (A) Structure of AG1024. (B) Effect of AG1024 on PANC-1 cell viability in normal medium, DMEM (●) and nutrient-deprived medium, NDM (○). PANC-1 cells incubated in DMEM for 24 h. The cells were then washed with PBS and the medium was replaced with either fresh DMEM or NDM. The indicated concentrations of AG1024 were added to each well and the cells were incubated for 24 h. Cell viability was determined using the MTT assay. (C) Flow cytometric analysis of AG1024-treated PANC-1 cells. PANC-1 cells were cultured with 0.3 μM AG1024 in DMEM or NDM for 12 h. The cells were stained with annexin V-FITC and propidium iodide according to instructions for the apoptosis detection kit and then analyzed using a flow cytometer. (D) Effect of conventional anticancer drugs on survival of PANC-1 cells in nutrient-deprived conditions. PANC-1 cells were incubated with indicated concentrations of doxorubicin, 5-fluorouracil and paclitaxel in DMEM (●) or NDM (○) for 24 h.

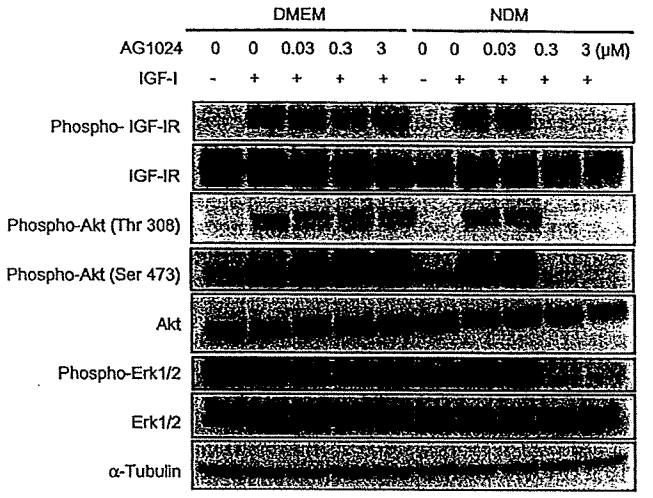

Fig. 2. Effect of AG1024 on IGF-1R activation. PANC-1 cells incubated in DMEM for 24 h were washed with PBS and the medium was replaced with either fresh DMEM or NDM. The cells were incubated with the indicated concentration of AG1024 for 1 h before stimulation with 50 ng/ml IGF-1 for 10 min. Cell lysates were resolved using SDS–PAGE and transferred to membranes for western blotting with specific antibodies.

anti-phospho-Erk, or anti-tubulin antibodies. Horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG antibodies were used as secondary antibodies (GE Healthcare, Piscataway, NJ). The blots were developed using ECL reagent according to the manufacturer's instructions (GE healthcare).

Flow cytometric analysis. PANC-1 cells (5×10^5) in 60-mm dishes were incubated in DMEM for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM or NDM. AG1024 (0.3 μM) was then added and the cells were cultured for 12 h. The cells were incubated with annexin V-FITC and propidium iodide using an annexin V-FITC apoptosis detection kit (Biovision Research Products, Mountain View, CA) and analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Statistical analysis. All data are representative of three independent experiments with similar results. The statistical data are expressed as mean ± SD using descriptive statistics.

Results

AG1024 is preferentially cytotoxic to human pancreatic cancer PANC-1 cells in nutrient-deprived conditions

To identify cytotoxic agents that function preferentially on nutrient-deprived cells, we tested the cytotoxic effects of small-molecule inhibitors in the SCADS inhibitors kit I. As shown in Table S1, a specific inhibitor of IGF-1R tyrosine kinase, termed AG1024, was found to be cytotoxic to PANC-1 cells in nutrient-deprived medium (NDM), but not in normal medium (DMEM). The structure of AG1024 [22], otherwise known as 2-(3-bromo-5-*t*-butyl-4-hydroxybenzylidene)malonitrile, is shown in Fig. 1A. To determine the dose–response relationship of AG1024 cytotoxicity, PANC-1

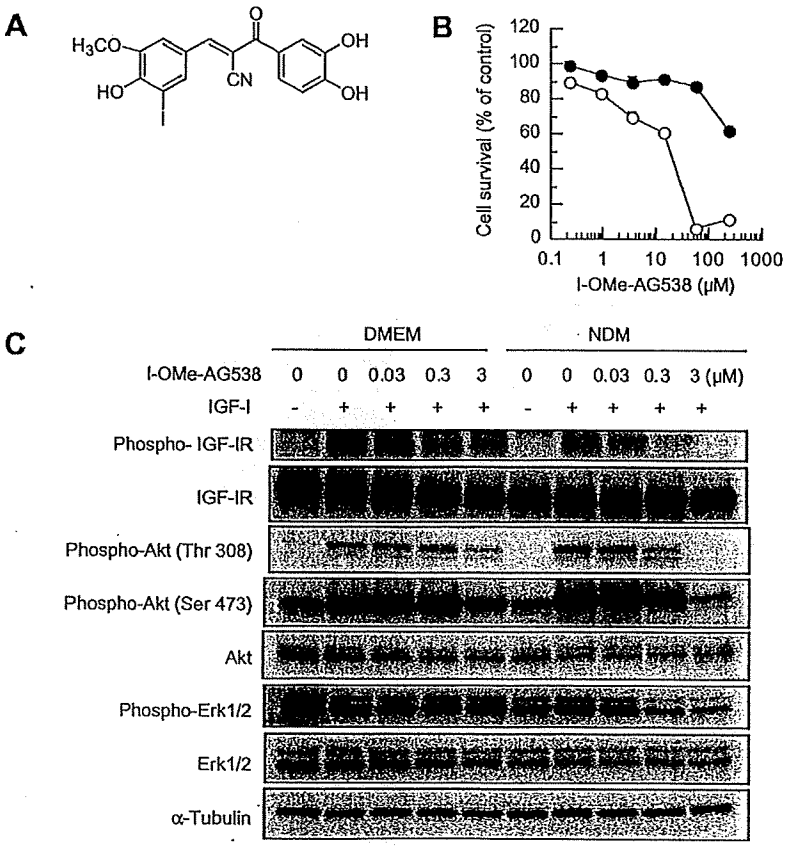

Fig. 3. Effect of I-OMe-AG538 on survival of nutrient-deprived PANC-1 cells. (A) Structure of I-OMe-AG538. (B) Effect of I-OMe-AG538 on PANC-1 cell viability in DMEM (●) or NDM (○). PANC-1 cells were incubated with the indicated concentrations of I-OMe-AG538 in DMEM or NDM for 24 h. (C) Effect of I-OMe-AG538 on IGF-1R activation. PANC-1 cells in DMEM or NDM were incubated with the indicated concentration of I-OMe-AG538 for 1 h before stimulation with 50 ng/ml IGF-1 for 10 min. Cell lysates were resolved using SDS–PAGE and transferred to membranes for western blotting with specific antibodies.

cells grown in NDM or DMEM were exposed to increasing concentrations of AG1024 for 24 h (Fig. 1B). The cytotoxic effect of AG1024 was more than 100 times greater on nutrient-deprived PANC-1 cells (NDM IC_{50} 0.055 μM) relative to cells in nutrient-sufficient medium (DMEM IC_{50} 21 μM). In DMEM, 0.3 μM AG1024 did not induce any significant PANC-1 cell death as determined using propidium iodide and annexin V staining and flow cytometry (Fig. 1C). In contrast, 34% of the cells grown in NDM and treated with the same concentration of AG1024 showed propidium iodide-positive/annexin V-negative staining. We compared the cytotoxicity of AG1024 to that of several conventional anticancer drugs, including doxorubicin, 5-fluorouracil and paclitaxel, in PANC-1 cells grown in NDM versus DMEM (Fig. 1D). The cytotoxicity of doxorubicin, 5-fluorouracil and paclitaxel on PANC-1 cells grown in either medium for 24 h was significantly weaker than AG1024. These results demonstrate clearly that AG1024 exhibits preferential cytotoxicity to nutrient-deprived PANC-1 cells.

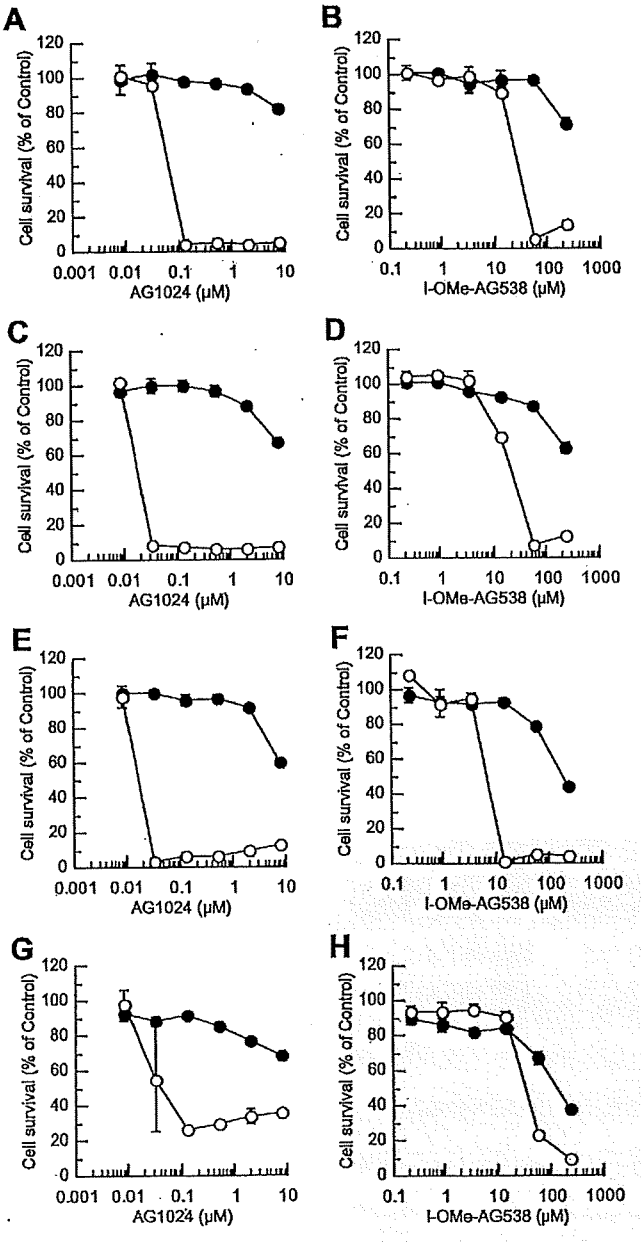

Fig. 4. Effect of AG1024 and I-OMe-AG538 on various human pancreatic cancer cell lines in nutrient-deprived conditions. Human pancreatic cancer cells were incubated with indicated concentrations of AG1024 or I-OMe-AG538 in DMEM (●) or NDM (○) for 24 h. A,B, Capan-1; C,D, MIA Paca-2; E,F, BxPC-3; G,H, PSN-1.

AG1024 inhibits activation of IGF-1R in nutrient-deprived PANC-1 cells

Because AG1024 is a specific inhibitor of IGF-1R kinase, we examined the effect of AG1024 on IGF-1-mediated phosphorylation of IGF-1R in PANC-1 cells grown in different media (Fig. 2). While addition of 0.3 μM AG1024 to PANC-1 cells grown in NDM resulted in a complete inhibition of IGF-1R autophosphorylation, phosphorylation of IGF-1R was only weakly inhibited in cells grown in DMEM with 10-fold higher concentrations of AG1024 (3 μM). AG1024 also inhibited the phosphorylation of Akt (Thr 308), Akt (Ser 473) and Erk, which normally occur as a result of IGF-1R activation. These results demonstrate that AG1024 is a potent inhibitor of IGF-1R activation in nutrient-deprived PANC-1 cells.

I-OMe-AG538 is preferentially cytotoxic to nutrient-deprived PANC-1 cells

In testing whether other IGF-1R inhibitors also functioned preferentially in nutrient-deprived cells, we found that I-OMe-AG538 [23] (another specific inhibitor of IGF-1R, Fig. 3A) also was more cytotoxic to cells in nutrient-deprived medium relative to those in nutrient-sufficient conditions (Fig. 3B). The effect of I-OMe-AG538 on IGF-1R activation in nutrient-deprived cells was similar to AG1024, in that it blocked phosphorylation of IGF-1R, Akt and Erk (Fig. 3C). Our results demonstrate clearly that the IGF-1R inhibitors AG1024 and I-OMe-AG538 both inhibit IGF-1R-mediated signaling and are preferentially cytotoxic to nutrient-deprived PANC-1 cells.

Inhibitors of IGF-1R show preferential cytotoxicity to various human pancreatic cancer cells lines in nutrient-deprived conditions

To determine whether inhibitors of IGF-1R kinase exhibit preferential cytotoxicity to other nutrient-deprived human pancreatic cancer cell lines, we examined the cytotoxic effects of AG1024 and I-OMe-AG538 on Capan-1, MIA Paca-2, BxPC-3, and PSN cells (Fig. 4). AG1024 and I-OMe-AG538 were significantly more cytotoxic to all four human pancreatic cancer cell lines in NDM relative to DMEM, indicating that the cytotoxicity of IGF-1R kinase inhibitors is likely to occur in nutrient-deprived human pancreatic cancer cells. To understand the specificity of IGF-1R kinase inhibitors, we also examined the cytotoxic effects of other representative receptor tyrosine kinase inhibitors (Fig. S1). The cytotoxicities of AG1296 (a PDGFR kinase inhibitor) [24] and AG1478 and PD168393 (EGFR kinase inhibitors) [25–27] were significantly reduced, relative to IGF-1R inhibitors, in both nutrient-deprived and -fed PANC-1 cells. These results indicate that specific inhibition of IGF-1R kinase is important in promoting preferential cytotoxicity in nutrient-starved human pancreatic cancer cells.

Discussion

Tumor microenvironment strongly influences tumor growth and progression. Many aspects of physiology that differentiate solid tumors from normal tissues arise from differences in vasculature. Disorganized vascular systems in tumors result in large areas of tumor exposed to nutrient starvation and hypoxic conditions. In addition, due to the unregulated growth of tumor cells caused by genetic and epigenetic alterations, cells proliferate more rapidly than normal cells and nutrient and oxygen demands often exceeds supply [28–30]. Cancer cells, in particular highly aggres-