Growth-related changes of phosphorus metabolites in VX-2 carcinoma implanted into rabbit thigh muscle: in vivo $^{31}$P MR spectroscopy

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Received: 15-06-2015 Accepted: 21-06-2016 DOI:10.18805/ijar.v0i0f.3798

ABSTRACT
The purpose of this study was to evaluate the usefulness of in vivo $^{31}$P magnetic resonance spectroscopy (MRS) for monitoring changes in growth-related phosphate metabolite concentration and intracellular pH value in rabbit thigh muscle implanted with VX-2 carcinoma. The time-course magnetic resonance imaging (MRI) and in vivo $^{31}$P MRS were examined weekly in the course of 10 weeks following the onset of a VX-2 carcinoma implantation. The spectra were quantitatively analyzed to obtain vital information on the time course variation of the phosphorus metabolites and intracellular pH value according to the tumor growth. Elevation in the concentrations of phosphoromonoesters (PME), inorganic phosphate (Pi), and phosphodiesters (PDE) was observed over the time course of 3-4 weeks after the implantation of VX2 carcinoma, while the rest of the metabolites, PCr and ATP tended to be constant. The concentration changes of PME, Pi, and PDE were positively correlated with the volumes of tumor necrosis. The intracellular pH values decreased with the time course of tumor growth and the volumes of tumor necrosis. In vivo $^{31}$P MRS is capable of non-invasive monitoring of intracellular pH values as well as the concentration changes of phosphate metabolites during tumor growth.

Key words: In vivo $^{31}$P magnetic resonance spectroscopy (MRS), Neoplasm, Phosphorus metabolites, VX-2 carcinoma.

INTRODUCTION

VX-2 carcinoma grows rapidly with local infiltration and has many morphologic, biochemical and biologic features in common with human carcinoma (Young et al. 1976, Mills et al. 1988). Especially, VX2 tumors of rabbits are similar to human head and neck squamous cell carcinoma, liver cancer, and other solid tumors with invasion and lymph node metastasis (Lee et al. 2009).

In MR imaging (MRI) studies, the morphologic characteristics of the VX-2 carcinoma, based on signal intensities, revealed the tumor to be moderately firm and lobulated with little capsule formation, and the tumor frequently has necrotic and cystic central areas (Young et al. 1976, Choi et al. 1993). Together with rapid tumor growth, necrosis can be used to predict the tumor grade (Dean et al. 1990) or prognosis after chemotherapy (Sironi et al. 1991). However, there has been little correlation between tumor growth and necrosis with conventional MRI indices of viability, and furthermore, conventional MRI is limited to quantify the metabolic changes according to tumor development.

The biochemical and metabolic changes within tumor cells precede the gross morphologic or pathologic changes during the period of tumor growth or response to therapy. MR spectroscopy (MRS) is capable of monitoring and quantifying the biochemical changes of metabolites in various tissues and organs at the cellular level (Sijens et al. 1988, Kim et al. 2015, Kim et al. 2016). Especially, 31-P MRS has been used to study high energy phosphate metabolism associated with a variety of pathologies in humans or animal models (Mills et al. 1988, Sironi et al. 1991, Choi et al. 2008, Lee et al. 2009). Up to date, there have been few studies focusing on the phosphorus metabolite changes accompanied by tumor growth by using in vivo $^{31}$P MRS (Mills et al. 1988), and the biochemical changes underlying tumor development have not been clearly elucidated.

The aim of this study was to assess the usefulness of in vivo $^{31}$P MRS for monitoring the changes of growth-related phosphorus metabolite concentration and intracellular pH values in the VX-2 carcinoma implanted into the thigh muscles of rabbits.

MATERIALS AND METHODS

Animal procedures and implantation of VX-2 carcinoma:
All procedures in this study were conducted with the approval of the animal research committee at Chonnam National University Hospital.

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A group of 30 rabbits weighing 2-3kg (mean 2.7kg) were examined for this study. Each animal was maintained in a single cage at a controlled temperature of 22±2°C, humidity of 40-60%, air ventilation of 10-20 times/hr and photoperiod of 12 hr light with 150-300Lux/12 hr darkness. Thirty rabbits were separated into ten groups and each group contained 3 rabbits. One group was MR scanned prior to the implantation of VX2 carcinoma and then was secondly scanned at the first week following the implantation of VX2 carcinoma, followed by extraction of the tumor tissues from the 3 rabbits. The other 9 groups were MR scanned weekly from the 2nd week through the 10th week, where each group was sacrificed after MR examination.

The procedure for the implantation of VX-2 carcinoma was as follows: The rabbits were sacrificed with an intramuscular injection of ketamine (25.0 mg/kg body weight) followed by an intravenous injection of sodium pentobarbital (200 mg/kg body weight). The tumor tissues in the thigh muscle were extracted and the intratumoral necrosis regions were eliminated. The thin layer of peripheral tumor tissues was dissected from the remnant tissues without necrosis and the dissected tumor tissues were fragmented in a suspension by scissors. The tumor suspension was loaded into a 1mL tuberculin injector using an 18 gauge intravenous cannula (Medicut®, Jelcco, San Francisco, USA) or the needle used for angiography (Cook Inc., Bloomington, USA). Then, the tumor cells were implanted by the percutaneous injection of 0.5 mL suspension into the rabbit’s thigh muscles.

Magnetic resonance imaging (MRI): All MR examinations were performed on a 1.5T Signa Twin speed MR scanner (GE Healthcare, Milwaukee, USA) using a flat-type GP surface coil with 8-inch (transmit) and 5-inch (receive) diameter. The T1-weighted images (T1WI) (TR/TE= 400/9ms) and T2-weighted images (T2WI) (TR/TE= 3200/102 ms) were acquired with field of view (FOV)= 16×16 cm², number of excitation (NEX)= 2, acquisition matrix= 256×192, slice thickness= 5 mm, slice gap= 2.5 mm and total scan time= about 15 minutes (Fig. 1).

In vivo 31P MR spectroscopy (MRS): The single-voxel in vivo 31P MRS was performed using a free induction decay chemical shift image (FIDCSI) pulse sequence with TR= 5000ms, NEX= 192, spectral width= 5000Hz, number of data points= 2048 and total scan time= about 16 minutes.

Rabbits were posed to the right lateral decubitus position to acquire spectra from the tumor lesions, and then were changed to the left side to obtain spectra from the normal thigh muscle. The voxel size for normal thigh muscle was 24 (=4×3×2) cm³ (Fig.1a), while the voxel size for tumor lesion varied with the growth of VX-2 carcinoma, for example, 21 (=3.5×3×2) cm³ at the 1st week and 84 (=7×6×2) cm³ at the 10th week.

Data processing and statistical analysis: The 31P MR spectra were post-processed and analyzed by means of the MR spectroscopic analysis package in the SAGE program (GE Healthcare, Milwaukee, USA). All the metabolites were assigned with respect to the chemical shift of phosphocreatine (PCr) which was used as the reference (PCr= 0.00 parts per million (ppm)): phosphomonoesters (PME, 6.7ppm), inorganic phosphate (Pi, 5.1ppm), phosphodiesters (PDE, 3.1ppm), PCR (0.0ppm), g-phosphorus of adenosine triphosphate (γ-ATP, -2.2ppm), α-ATP (-7.6ppm), and β-ATP(-16.0ppm). For the relative quantification of the metabolites, the β-ATP resonance peak was used as an internal reference. In addition, intracellular pH values were calculated from the chemical shift difference between the PCr and Pi peaks (Gerweck 1998).

The correlations between the necrosis volumes and the levels of 31P metabolites or the intracellular pH value were assessed by using linear and high-order regression models (SPSS version 19.0 Inc., IBM Company, Illinois, USA).

RESULTS AND DISCUSSION
Time-course changes of the morphology and 31P metabolic concentration in VX2tumors: Figure 1 shows the time-course changes of the 1H MR images and 31P MR spectra which were acquired every 2 weeks following VX-2 implantation, and a week-based changes of the morphology and the levels of 31P metabolites are summarized in Tables 1 and 2, respectively. Figure 2 is the three dimensional scatter diagram showing the time-course changes of the tumor size (cm) and its necrotic portion (%). The size of the tumors and the volume of the necrotic portions in tumors proportionally increased with tumor growth as follows: 2.5 cm and 4.5% at 1 week after implantation of VX-2 carcinoma, 2.9 cm and 23.2% at 2 weeks, 4.4 cm and 24.0% at 3 weeks, 5.6 cm and 35.8% at 4 weeks, 7.6 cm and 47.9% at 5 weeks, 7.7 cm and 52.9% at 6 weeks, 8.3 cm and 54.5% at 7 weeks, 8.6 cm and 58.2% at 8 weeks, 11.5 cm and 82.0% at 9 weeks, and 11.9 cm and 86.1% at 10 weeks, respectively. At 1-2 weeks, the nests of the tumor cells showed inhomogeneous high signal intensities on T2WI compared to normal muscles. Surrounding fibrosis irregularly showed the inhomogeneous signal intensities with abundant vascularity on T1WI and T2WI. Especially, the necrosis regions in the central portion were well observed with high signal intensities on T2WI (Fig. 1d-f). It should be noted that the VX-2 carcinoma typically underwent liquefactive necrosis as it grew.

The right panel in Figure 1 shows the representative 31P MR spectra acquired every 2 weeks, and the time-course variation of 31P metabolic concentrations is summarized in Table 2. Figure 3 demonstrates the time-course concentration changes of 31P metabolites. The levels of PME/β-ATP (Fig. 3a), Pi/β-ATP (Fig. 3b) and PDE/β-ATP (Fig. 3c) increased from 2-4 weeks to 10 weeks after VX-2 implantation. The γ-ATP/β-ATP (Fig. 3e) and α-ATP/β-ATP (Fig. 3f) were
Fig 1: Time-course changes of T2WI images (left) and localized in vivo $^{31}$P MR spectra (right), which were respectively acquired from normal (a) and tumor lesions at 2 weeks (b), 4 weeks (c), 6 weeks (d), 8 weeks (e) and 10 weeks (f) following the onset of VX-2 implantation in the right thigh muscle of a rabbit.
constant in a given time period. However, PCr/β-ATP (Fig. 3d) showed variation.

**Correlation between the necrosis volume and the concentration of 31P metabolites:** Figure 4 showed the correlation between the necrosis volumes and the level of 31P metabolites, and the correlation coefficients are summarized in Table 3. The necrosis volumes were positively correlated with PME (Pearson’s $r = 0.949; p$-value= 0.000), PDE ($r = 0.851; p = 0.001$), and Pi ($r = 0.683; p = 0.021$) and were not correlated with α-ATP ($r = 0.146; p = 0.667$), PCr ($r = -0.080; p = 0.814$) and γ-ATP ($r = 0.059; p = 0.862$).

Figure 5 showed the correlations of the intracellular pH values with the tumor growth (a) and the necrosis volumes (b). The intracellular pH values were negatively correlated with the growth of VX-2 carcinoma ($r = -0.745; p = 0.008$) and the necrosis volume ($r = -0.715; p = 0.013$). The pH values continuously decreased with an increment of the necrotic portion during the time period of tumor growth.

Considering the pathological features, VX-2 tumor had 3 layers which consisted of a tumor cell layer of severe vascularity, a connective tissue layer with abundant vascularity in the peripheral marginal zone and a necrotic tissue layer at central portion (Choi et al. 1993). In our study, the specific features in the nests of tumor cells, connective tissues and necrotic tissues were distinctively observed during growth of the VX-2 tumor. The changes in the tumor tissues were accompanied with hemorrhage, surrounding fibrosis and necrosis. The tumoral tissues lost homogeneity by hypervascularity and surrounding fibrosis, and the signal intensities of T1WI and T2WI began to change inhomogeneously with tumor cell growth. These findings were well concordant with the pathological changes in previous studies (Choi et al. 1993). Therefore, VX-2 carcinoma, which can be used in experimental tumor model, provided sufficient accuracy and reliability to estimate tumor-growth; and the distinctive features of VX-2 carcinoma evolution will provide predictability at each stage.

**Table 1:** Time-course changes of tumor size and necrotic portion in rabbits with VX-2 carcinoma

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Number of cases</th>
<th>Tumor size* (cm)</th>
<th>Necrotic portion (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>2.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>2.9</td>
<td>23.2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>4.4</td>
<td>24.0</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>5.6</td>
<td>35.8</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>7.6</td>
<td>47.9</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>7.7</td>
<td>52.9</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>8.3</td>
<td>54.5</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>8.6</td>
<td>58.2</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>11.5</td>
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</tr>
<tr>
<td>10</td>
<td>3</td>
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</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
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*Maximum diameter of tumor size
An interesting finding of in vivo $^{31}$P MRS was that the PME and PDE peaks continuously increased from 3 weeks to 10 weeks. The intracellular pH values gradually decreased with tumor growth. During the 1-2 weeks, the average levels of $^{31}$P metabolites were in the ranges of normal tissues. In these time periods, the metabolic changes reflected that the vascular blood supply is adequate and aerobic metabolism dominates. These metabolic changes at these time periods were consistent with the $^{31}$P-NMR spectral patterns acquired from the early stage in rabbits implanted with VX-2 carcinoma (Mills et al. 1988). During the 3-4 weeks, the concentration of PME, Pi and PDE increased as compared to the levels in normal tissues, whereas PCr concentration and intracellular pH value decreased. During 5-7 weeks, the concentration of PME and PDE continuously increased, whereas intracellular pH value continuously decreased. However, the levels of Pi and PCr were sustained. In these time periods, the increased values of PME and PDE are important metabolic changes because the PME and PDE metabolites are involved in synthesis and/or metabolism of phospholipid, and especially PME is accumulated by glycolysis as glycolytic intermediates. Also, decreasing intracellular pH value in tumor tissues reflected to increase the acidic metabolite such as lactate, a product of glucose metabolism under the anaerobic condition. Therefore, tumor tissues became hypoxic, suggesting prominent anaerobic glycolysis. During 8-10 weeks, the levels of PME, Pi, PDE and PCr greatly increased, whereas the intracellular pH value decreased. These findings suggested that the peaks of PME and PDE could be used as indices of a tumor growth with the degree of necrosis. In addition, the intracellular pH value could characterize different physiological conditions indicating the status of oxygen supply in the metabolism of glucose.

The PME, PDE and Pi were positively correlated with necrosis volumes. Also, the concentration levels of PME and PDE predominantly increased with tumor cell growth. However, ATP metabolites in the time periods were constant, and this means that the VX-2 tumor growth lead to changes of phospholipid metabolism, not energy metabolism. Daly et al. (1987) reported significant increments to PME and PDE as indices of tumor growth.
and PDE peaks in human breast cancer cells by using \( ^{31}P \) NMR spectroscopy. Aisen and Chenevert (1989) reported the elevation of PME peak in the in vivo \( ^{31}P \) MR spectra of various tumors. The PME and PDE indicated the significance of phospholipid precursors and catabolites as markers for tumor detection, malignancy, progression and the response to treatment (Si\( j \)ens et al. 1988, Podo 1999). The major metabolic compounds of PME are phosphocholine and phosphoethanolamine (Cousins 1995). These metabolites are the intermediates in the biosynthesis of phosphoglycerides and indicate the presence of precursors of cytoplasmic membrane synthesis (Si\( j \)ens et al. 1988). The complete deacylation of phospholipids produce glycerophosphocholine and glycerophosphoethanolamine (Daly et al. 1987). These metabolites are the major metabolic compounds of PDE, which indicate the presence of breakdown products of the membrane (Cousins 1995). Moreover, the ratio of PME/PDE can serve as an index of the metabolism of membrane phospholipids and reflect changes in the rate of membrane synthesis or metabolic turnover (Mills et al. 1988). Present findings support that the elevations of PME and PDE are reflected with the increments of cell membrane synthesis and cell differentiation speeds during growing VX-2 carcinoma cells. Therefore, this study can suggest that the PME peak could be used as “sensitive indicator” which represents cell membrane synthesis and tumor cell growth or treatment. And, the PME and PDE related to phospholipids could be considered as “indicators” reflecting the mechanisms of biochemical changes following the growth-related changes of VX-2 carcinoma.

Another significant finding of this study is the decrement of intracellular pH value as the tumor grew. The intracellular pH value was negatively correlated with necrosis volume. Also, the intracellular pH values in tumor tissues had the tendency of acidification. Human cancer researches (Adam and Bellomo 1997, Evelhoch 2001) have reported that the in vivo measurements of intracellular pH values have shown the microenvironment in tumors and their values differentiated tumoral tissues from normal tissues. The present study indicated that VX-2 tumor growth lead to an increment of Pi concentration with the reduction of Pi chemical shift in in vivo \( ^{31}P \) MR spectra, and consequently, tumor tissues showed intracellular acidification, leading to hypoxic states. However, several studies (Gerweck 1998, Evelhoch 2001) have reported that the intracellular pH estimated from \( ^{31}PMR spectrum was essentially equal or
Fig 4: Correlation between the necrosis volumes and the concentrations of phosphorus metabolites: (a) PME/β-ATP, (b) Pi/β-ATP, (c) PDE/β-ATP, (d) PCR/β-ATP, (e) γ-ATP/β-ATP and (f) α-ATP/β-ATP.

Fig 5: Correlations of intracellular pH values with growth of VX-2 carcinoma (a) and necrosis volume (b), in which the corresponding regression lines with 95% confidence band are shown.
slightly more basic (alkaline) in tumoral tissues relative to normal tissues. Therefore, the measurement of intracellular pH needs further investigation in order to be clarified.

It is noted that Ketamine was used to maintain anesthesia during this study. However anesthetic agents were not clearly investigated in their effects on concentrations of high energy phosphate metabolites. Despite having a total of 30 rabbits in the study group, this study has had the limitation of having a small sample size, only three rabbits in an MR examination every week. In addition, the peak of each metabolite started overlapping as the tumor grew and this generated the possibility of an erroneous estimation of intracellular pH values due to a chemical shift between PCr and Pi. Also, Mg$^{2+}$ concentrations, which are linked to changes in exercise capacity, immune function and disease, can be measured from the changes of chemical shift between α-ATP and β-ATP (Gupta and Yushok 1980), the Mg$^{2+}$ level was not measured because of the potential limitation for measuring the relatively small frequency shifts in the β-ATP peak in this study. Further studies are needed to confirm these findings in animal models for the treatment of VX-2 carcinoma.

In brief, changes of phosphorus metabolites (PME, Pi and PDE) and intracellular pH value were found to depend on the degree of the necrosis. The intracellular pH value was negatively correlated with necrosis volume, and it started to decrease as the tumor necrosis became severe. However, PCr, γ-ATP, α-ATP and β-ATP signals were relatively constant and found to be independent of the tumor growth and the degree of the necrosis.

It is concluded that in vivo 31P MRS is capable of noninvasively monitoring tumor growth by presenting quantitative information on phosphorus metabolites and intracellular pH. In addition, 31P MRS may demonstrate potential value in monitoring effects of tumor therapy in vivo.

ACKNOWLEDGMENT

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2015R1A2A2A01007827).

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