Abstract. The compound 3-bromopyruvate (3BP) is an analogue of pyruvate, which is the final product of glycolysis that enters the citric acid cycle. The present study aimed to investigate the suppressive effects of 3BP on the proliferation and motility of hepatocellular carcinoma (HCC) cells. HLF and PLC/PRF/5 cells were cultured with 3BP and subjected to an MTS assay. Apoptosis was analyzed by hematoxylin and eosin staining. Cell motility was analyzed using a scratch assay. Real-time quantitative polymerase chain reaction (PCR) was performed to determine the expression levels of cyclin D1 and matrix metalloproteinase (MMP)9. Proliferation of both cell lines was significantly suppressed by 3BP at 100 µM (P<0.05). The expression level of cyclin D1 was decreased after 3BP treatment at 100 µM in both cell lines (P<0.05). Pyknotic nuclei were observed in the cells cultured with 3BP at 100 µM. These results revealed that 3BP suppressed cell proliferation, decreased the expression of cyclin D1, and induced apoptosis in HCC cells. 3BP significantly suppressed motility in both cell lines (P<0.05). The expression level of MMP9 was significantly decreased (P<0.05). 3BP suppressed the proliferation and motility of HCC cells by decreasing the expression of cyclin D1 and MMP9.

Introduction

Hepatocellular carcinoma (HCC) is a common liver malignancy and its prognosis is poor despite advancements in treatment (1). The treatment options for HCC include local ablation, surgery, transcatheter arterial chemoembolization, and systemic chemotherapy (2,3). Molecular therapy has been under investigation for the development of a novel treatment for HCC (4).

Glycolysis is more pronounced in cancer cells than in normal cells. Cancer cells require more glucose under conditions of a sufficient oxygen supply (Warburg effect) (5). The Warburg effect is strong in cancer cells, but weak in normal cells. If glycolysis is inhibited, it is expected that cancer cells die while normal cells survive (6). Thus, inhibition of glycolysis is a new treatment strategy for HCC (7).

Pyruvate is the final product of glycolysis and enters the citric acid cycle (8). 3-Bromopyruvate (3BP) is an analogue of pyruvate (9) and is used as an inhibitor of glycolysis. 3BP inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase (10), and unlike other chemotherapeutic agents, it is less toxic to cells as it mimics pyruvate (9). Therefore, 3BP can be used for the treatment of cancer (11).

In the present study, we investigated the suppressive effects of 3BP on the proliferation and motility of HCC cells.

Materials and methods

Cell culture. HCC cell lines, HLF and PLC/PRF/5, were purchased from the Riken Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA). The cells were cultured in 10-cm dishes (Asahi Techno Glass, Funabashi, Japan) with 5% carbon dioxide at 37°C in a humidified chamber.

Cell proliferation assay. The cells were trypsinized, harvested, and spread on 96-well plates (Asahi Techno Glass) with 10,000 cells/well. The cells were cultured in DMEM supplemented with 10% FBS. 3BP was added at 0, 1, 3, 10, 30, or 100 µM to the culture medium. The cells were cultured for 72 h and subjected to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyoxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay, according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). MTS is reduced by cells to a colored formazan product with an absorbance maximum at 490 nm. The absorbance was measured using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA, USA).
ToM IzAWA et al.: ROLE OF 3BP IN HCC CELLS

Real-time quantitative polymerase chain reaction. Total RNA (5 µg), which was isolated using Isogen (Nippon Gene, Tokyo, Japan), was used for the first-strand cDNA synthesis with SuperScript III and oligo(dT) according to the manufacturer’s instructions (Life Technologies). Real-time quantitative PCR was performed using Fast SYBR Green Master Mix (Life Technologies) with Mini Opticon (Bio-Rad). The results were analyzed using the Mini Opticon system (Bio-Rad). Real-time quantitative PCR was performed for 40 cycles, with 5 sec of denaturation and 5 sec of annealing-extension. Table I shows primer sequences used. RPL19 was used as an internal control since it is a housekeeping gene that is constitutively expressed (12).

Scratch assay and hematoxylin and eosin staining. The cells were plated on 4-well chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA). When the cells reached confluence, they were scratched with a sterile razor. The cells were incubated with 3BP (0 or 100 µM) for 48 h and stained with hematoxylin and eosin. The cells were plated in 4-well chamber slides (Becton Dickinson) for the analysis of apoptosis. The

Table I. Primer sequences used for real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Description</th>
<th>Product size (bp)</th>
<th>Annealing temperature (˚C)</th>
<th>Cycle</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMC355</td>
<td>5’-AGAGGCGGAGGAGAACAAACAG-3’</td>
<td>Cyclin D1, forward</td>
<td>180</td>
<td>60</td>
<td>40</td>
<td>NM_053056</td>
</tr>
<tr>
<td>OMC356</td>
<td>5’-AGCAGTGATAGGACAGGAGGTG-3’</td>
<td>Cyclin D1, reverse</td>
<td>89</td>
<td>60</td>
<td>40</td>
<td>NM_004994</td>
</tr>
<tr>
<td>OMC749</td>
<td>5’-CCTGGCGAGATCCAAACCT-3’</td>
<td>MMP9, forward</td>
<td>89</td>
<td>60</td>
<td>40</td>
<td>NM_004994</td>
</tr>
<tr>
<td>OMC750</td>
<td>5’-GCAAGTCTTTCCAGTAGTTGGAT-3’</td>
<td>MMP9, reverse</td>
<td>89</td>
<td>60</td>
<td>40</td>
<td>NM_004994</td>
</tr>
<tr>
<td>OMC321</td>
<td>5’-CGAATGCGAGAGGTTCAC-3’</td>
<td>RPL19, forward</td>
<td>157</td>
<td>60</td>
<td>40</td>
<td>BC095445</td>
</tr>
<tr>
<td>OMC322</td>
<td>5’-CCATGAGATCCGCTTGT-3’</td>
<td>RPL19, reverse</td>
<td>157</td>
<td>60</td>
<td>40</td>
<td>BC095445</td>
</tr>
</tbody>
</table>

MMP9, matrix metalloproteinase 9; RPL19, ribosomal protein L (RPL) 19.

Figure 1. MTS assay. HLF (A) and PLC/PRF/5 cells (B) were subjected to an MTS assay (see Materials and methods) 72 h after incubation with 3-bromopyruvate (3BP) at 0, 1, 3, 10, 30, or 100 µM. Error bar, standard deviation, *P<0.05 compared with 0 µM, n=3.

Figure 2. Real-time quantitative PCR of cyclin D1. HLF (A) and PLC/PRF/5 cells (B) were incubated with 3-bromopyruvate (3BP) at 0, 10, 30, or 100 µM. Error bar, standard deviation, *P<0.05 as compared with 0 µM, n=3.
ONCOLOGY REPORTS 35: 59-63, 2016

cells were incubated with 3BP (0 or 100 µM) for 48 h and stained with hematoxylin and eosin. The stained slides were observed under an AX80 microscope (Olympus, Tokyo, Japan) for the analysis of apoptosis and scratch assay. For the scratch assay, the distance between the scratched line and the growing edges of the cells was measured at five different points.

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) and statistical analysis was carried out with JMP 10.0.2 software (SAS Institute, Cary, NC, USA). P-values <0.05 were determined to be statistically significant.

Results

To address the possibility that 3BP suppresses cell proliferation, HLF (Fig. 1A) and PLC/PRF/5 cells (Fig. 1B) were incubated with 3BP. After 72 h of incubation, the cells were subjected to MTS assay. Proliferation of both cell lines was significantly suppressed (P<0.05).

The expression levels of cyclin D1 were analyzed with real-time quantitative PCR, as this gene plays a role in cell proliferation (Fig. 2) (13). The expression levels of cyclin D1 were decreased in both cell lines (P<0.05).

We hypothesized that apoptosis may be involved in the suppression of cell proliferation. To clarify the role of apoptosis, hematoxylin and eosin staining was performed in the HLF (Fig. 3A and B) and PLC/PRF/5 cells (Fig. 3C and D) after a 48-h incubation with 3BP at 0 µM (Fig. 3A and C) or 100 µM (Fig. 3B and D). Pyknotic nuclei were observed in the cells cultured with 3BP at 100 µM (arrows).

In order to investigate cell motility, a scratch assay was performed in the HLF (Fig. 4A and B) and PLC/PRF/5 cells (Fig. 4C and D) after a 48-h incubation with 3BP at 0 µM (Fig. 4A and C) or 100 µM (Fig. 4B and D). The distance between the growing edges of the cells and the scratched line was measured (Fig. 4E). Cell motility was significantly suppressed in both cell lines (P<0.05).
3BP reduces HCC cell activity and induces apoptosis (15). Ganapathy-Kanniappan et al found that 3BP at 150 µM suppressed the proliferation of Hep3B cells (16). In our study, 3BP at 100 µM induced the apoptosis of HLF and PLC/PRF/5 cells. Although the cells were different, the concentration of 3BP was almost the same between the previous report and our study. These reports and our data suggest that 3BP induces apoptosis in HCC cells at 100-150 µM.

Our results clearly showed that proliferation of HCC cells was suppressed by 3BP, and 3BP also inhibited the cell cycle (17). No reports exist on the effects of 3BP on cyclin D1 expression. In the present study, the expression of cyclin D1 decreased after 3BP treatment. This result indicated that cyclin D1 was involved in the suppression of cell proliferation by 3BP. However, the detailed mechanism underlying the decrease in the expression of cyclin D1 is yet to be elucidated.

3BP in combination with 2-deoxyglucose, which is another inhibitor of glycolysis, was found to suppress the motility of breast cancer cells (18). In the present study, 3BP alone successfully suppressed the motility of HLF and PLC/PRF/5 cells. Moreover, the expression levels of MMP9 decreased.

The previous report and our results clearly showed that 3BP suppressed the motility of cancer cells by decreasing the expression of matrix metalloproteinase with or without the combination of other agents.

One major limitation of 3BP is that it exerts limited antitumor effects in in vivo animal models (19). To overcome this limitation, a combination of 3BP and other agents could be employed (20). Furthermore, it is proposed that 3BP should be administered to patients in combination with other chemotherapeutic agents during transcatheter arterial chemobolization (21). No harmful effects were reported in an animal model (22).

In conclusion, 3BP suppressed the proliferation and motility of HCC cells by decreasing the expression of cyclin D1 and matrix metalloproteinase.

References


Discussion

MMP9 is involved in cell motility (14). RNA was isolated from the HLF (Fig. 5A) and PLC/PRF/5 cells (Fig. 5B) and subjected to real-time quantitative PCR. The expression levels of MMP9 were significantly decreased in both cell lines (P<0.05).


