Small Interfering RNA (siRNA) against the Bcl-2 Gene Increases Apoptosis in a Canine Melanoma Cell Line

Yuzuru WATANABE1), Rui KANO3)*, Haruhiko MARUYAMA1), Atsuhiko HASEGAWA2) and Hiroshi KAMATA1)

1)Department of Pathobiology, Nihon University School of Veterinary Medicine 1866, Kameino, Fujisawa, Kanagawa 252–8510 and 2)Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

(Received 2 April 2009/Accepted 16 November 2009/Published online in J-STAGE 9 December 2009)

ABSTRACT. The effects of down-regulation of Bcl-2 expression, by small interfering RNA (siRNA) against the canine Bcl-2 genes, on apoptosis were investigated by transfecting MCM-N1 (canine malignant oral melanoma cell line) cells with siRNA using cationic liposomes. The siRNA against the canine Bcl-2 genes increased the number of apoptotic cells. In addition, sequence-specific down-regulation of Bcl-2 expression was measured by RT-PCR and western blot analysis. The siRNA directed against these genes reduced both mRNA and protein expression in the MCM-N1 cells. Our study suggests the importance of Bcl-2 in canine melanoma tumors for inducing apoptosis and reinforces using Bcl-2 as a putative therapeutic target in canine malignant melanoma tumor.

KEY WORDS: apoptosis, Bcl-2, canine malignant melanoma, small interfering RNA (siRNA).

NOTE

Small Interfering RNA (siRNA) development [11] has become the most widely used technology for gene knockdown. When long double-stranded (ds) RNA molecules are introduced into plants and invertebrates, they are processed by the endonuclease direct into 21- to 23-nucleotide small RNAi. The small interfering RNA (siRNA) are then incorporated into the multicomponent for RNA-induced silencing complex (RISC), which unwinds the duplex and uses the ds strand as a guide to seek and degrade homologous mRNAs [4]. In our previous study, we showed that siRNA directed against the Bcl-2 gene reduced its mRNA and protein expression in the mammary gland tumor cell line CF33 [9], and that the number of viable cells decreased with an increase in the apoptotic cell rate [9].

In the present study, we evaluated siRNA compounds targeting Bcl-2 as a novel approach to downregulate Bcl-2 in canine melanoma cells. We found that a specific siRNA led to a moderate increase in apoptotic cell death and inhibition of cell growth.

Canine malignant melanoma is a spontaneous, highly aggressive neoplasm that can readily metastasize to other organs [8, 12]. Since systemic chemotherapy, surgical excision and radiation therapy for canine malignant melanoma has had poor outcomes [12], effective therapies are still required. Several novel therapeutic modalities have been reported, including methods for enhancing immunosurveillance (e.g., tumor vaccines and DNA vaccines) [5, 8, 12]. Immunomodulation therapy is currently an active tool in veterinary oncology; however, more effective therapy is required.

Bcl-2 is a strongly anti-apoptotic protein that is expressed in advanced human melanomas [7]. Different types of cellular damage as well as cellular dysregulation such as oncogene activation induce cell-intrinsic proapoptotic pathways [2]. Highly active is the mitochondrial pathway characterized by a release of micchondrial proapoptotic factors into the cytoplasm [2]. This step is critically controlled by the Bcl-2 [2]. Bcl-2 is critical regulators of mitochondrial membrane permeability and the proapoptotic mitochondrial pathway [2]. On the other hand, a role for Bcl-2 in the development of normal melanocyte stem cells is suggested by observation that Bcl-2 knock-out mice lose their dark hair color after birth owing to rapid degeneration of hair bulb melanocytes [7]. In melanoma cells, the malignant counterparts of melanocytes, Bcl-2 expression is found in up to 100% of human cases [7].

Because of the ability of this protein to decrease the apoptotic response to cytotoxic chemotherapy, Bcl-2 in human melanomas has been a target for anti-Bcl-2 small interfering RNA (siRNA) development [11]. Thus, canine Bcl-2 may be an important therapeutic target in canine melanoma.

The RNA interference (RNAi) has become the most widely used technology for gene knockdown. When long double-stranded (ds) RNA molecules are introduced into plants and invertebrates, they are processed by the endonuclease direct into 21- to 23-nucleotide small RNAi. The small interfering RNA (siRNA) are then incorporated into the multicomponent for RNA-induced silencing complex (RISC), which unwinds the duplex and uses the ds strand as a guide to seek and degrade homologous mRNAs [4]. In our previous study, we showed that siRNA directed against the Bcl-2 gene reduced its mRNA and protein expression in the mammary gland tumor cell line CF33 [9], and that the number of viable cells decreased with an increase in the apoptotic cell rate [9].

In the present study, we evaluated siRNA compounds targeting Bcl-2 as a novel approach to downregulate Bcl-2 in canine melanoma cells. We found that a specific siRNA led to a moderate increase in apoptotic cell death and inhibition of cell growth.

The canine malignant oral melanoma cell line MCM-N1 was purchased from DS Pharma Biomedical Co., Ltd (Fukita, Osaka, Japan) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% FBS, at 37°C in a humidified atmosphere containing 5% CO2.

The siRNA targeting the specific sequence 5'-CCA UGC AGC CUC UGU UUG A-3' (GenBank accession no. AB154172; 1046-1064 bp) was selected to down-regulate Bcl-2 expression in this study based on its previous effectiveness [9]. A non-targeting RNA (ntRNA) sequence (5'-UUC UCC GAA CGC GUC ACG U-3'), which does not target any known mammalian gene [1], was used as the negative control. The siRNA and ntRNA were synthesized using the Silencer siRNA Construction Kit (Life Technologies Inc., Carlsbad, CA, U.S.A.) according to manufacturer’s instructions.

*Correspondence to: KANO, R., Present address: Department of Pathobiology, Nihon University School of Veterinary Medicine, 1866 Kameino, Fujisawa, Kanagawa 252–8510, Japan. e-mail: kano@brs.nihon-u.ac.jp
Transfection was performed according to our previous report [9]. Transfection of MCM-N1 cells with siRNA and ntRNA was performed using cationic liposomes (siLentFect reagent, Bio-Rad Laboratories, Hercules, CA, U.S.A.) at approximately 50% confluency in 6-well plates, according to the manufacturer’s instructions. The mock transfection contained only the reagent (cationic liposomes). The transfected cells were examined using analysis of apoptosis assay and RT-PCR. All experiments were performed in triplicate. The data for the apoptosis analysis and RT-PCR are represented as mean ± SEM (standard error of the mean) and were analyzed by one-way analysis of variance (ANOVA) and corrected for multiple comparisons using the Bonferroni method. Statistical significance (p<0.05) was determined using the Excel Statistical Program File ystat2002 (Igaku Tosho Shuppan, Tokyo, Japan) software.

After 24 and 48-hr transfection, MCM-N1 cells were washed, collected and dropped on slides using cytoospin, respectively. Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method. The TUNEL method was performed according to the protocol of the DeadEnd™ Colorimetric Apoptosis Detection System (Promega Corp., Madison, WI, U.S.A.). At least 500 cells were counted by light microscopy. Apoptotic cells were identified according to the criteria of Oguma [10].

The proportion of apoptotic cells was determined by TUNEL method for ssDNA and was as follows immediately after the 48-hr transfection: anti-Bcl-2 siRNA transfection, 28.8 ± 3.4% (significantly higher than ntRNA transfection, P<0.05); ntRNA transfection, 1.7 ± 0.9%; mock transfection, 1.4 ± 0.5%; and medium alone, 0.8 ± 0.4% (Fig. 1).

After the 48-hr transfection, total RNA was extracted from the cells using the RNaseasy Mini Kit (QIAGEN) immediately. Reverse transcription was carried out by incubating 1 μg of total RNA, 0.5 μg of oligo (dT) primer and Omnirect Reverse Transcriptase enzyme (QIAGEN) at 42°C for 90 min and 95°C for 5 min, followed by incubation at 72°C for 15 min. Real time PCR for Bcl-2 and GAPDH (glycer-aldehyde-3-phosphate dehydrogenase) was performed using specific pairs of primers. The primer sequences and gene accession numbers are as follows: Bcl-2 (Forward: 5’-TGG ATG ACT GAG TAC CTG AA and Reverse: 5’-GCC CTA CTG ACT TCA CTT AT, AB116145) and GAPDH (Forward: 5’-GGG GAA AGC TGC CAA ATA TG and Reverse: 5’-ACC AGG AAA TGA GCT TGA CA, AB038240). GAPDH was used as an internal control for PCR amplification [6]. All PCR reactions were performed using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) and the following thermal program: initial denaturation at 95°C for 10 sec, followed by 35 cycles of denaturation at 95°C for 5 sec, annealing at 62°C (Bcl-2) or 60°C (GAPDH) for 20 sec and extension at 72°C for 20 sec. The relative concentration of PCR products derived from each target gene was calculated using the software of the LightCycler System. All experiments were performed in triplicate.

Bcl-2 mRNA expression significantly decreased in cells transfected with siRNA, whereas there was no change in cells transfected with ntRNA, either in mock reactions or in medium alone (Fig. 2a). Therefore, this siRNA down regulated Bcl-2 mRNA in MCM-N1 cells.

After the 48-hr transfection, cells were detached by trypsin treatment immediately, washed with PBS (Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) and then lysed at 4°C in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 mM EDTA] supplemented with complete miniprotease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). The cell debris was removed by centrifugation at 13,200 rpm for 5 min. Protein concentration was determined with the BCA protein kit (Roche Molecular Biochemicals, Mannheim, Germany). Each supernatant containing 15 μg of protein was boiled for 5 min in SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% sodium dodecyl sulfate, 0.01% bromophenol blue) containing 5% β-mercaptoethanol. The proteins were electrophoresed on a 12.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred on poly-vinylidene difluoride (PVDF) membranes (Bio-Rad) by semi-dry electrophoretic transfer. Membranes were blocked overnight with 5% non-fat milk in TBST (20 mM Tris-HCl pH 7.2, 0.3 M NaCl, 0.1% Tween 20) and then cut into two pieces. One piece was incubated with mouse anti-Bcl-2 monoclonal antibody (1:500 dilution, 610539, BD Transduction Laboratories, San Jones, CA, U.S.A.) for over night at 4°C. After washing with TBST (20 mM Tris HCl, pH 7.2, 0.3 M NaCl, 10.1% Tween 20), the membrane was then incubated with the goat anti-rabbit IgG secondary antibody (1:1000 dilution, A3562, Sigma-Aldrich) for 2 hr at 37°C. The other mem-

![Fig. 1. The proportion of apoptosis cells after transfection with siRNA, ntRNA, mock and medium alone. The proportion of apoptotic cells was determined by TUNEL method. Data represent mean ± SEM. “●” indicates anti-Bcl-2 siRNA (siRNA) transfection. “△” indicates non-targeting RNA (ntRNA) transfection. “○” indicates mock transfection containing only reagent (cationic liposomes); ■ indicates cultured in medium (Medium).](image-url)
brane piece was incubated with anti-β-actin monoclonal antibody (1:5000, Sigma-Aldrich) [3] for 2 hr at 37°C, washed with TBST (20 mM Tris HCl, pH 7.2, 0.3 M NaCl, 0.1% Tween 20), and then incubated with the secondary antibody (1:5,000 dilution, A3652, Sigma-Aldrich) under the same conditions as for anti-Bcl-2 antibody and using β-actin as an internal control to ensure equal protein extraction from cells. After washing with TBST, immunoreactive proteins were detected using alkaline phosphatase (NBT/BCIP Tablets, Roche Diagnostics Corporation, Basel, Switzerland).

Bcl-2 protein expression was determined by western blot analysis. Bcl-2 protein expression decreased in cells transfected with siRNA, whereas there was no change in cells transfected with ntRNA, either in mock reactions or in medium alone (Fig. 2b). Therefore, this siRNA inhibited expression of Bcl-2 protein in MCM-N1 cells.

High expression of anti-apoptotic genes, such as Bcl-2, commonly found in human cancers, contributes to neoplastic cell expansion and inhibits the therapeutic action of many chemotherapeutic drugs [11]. The siRNA against the canine Bcl-2 gene increased the apoptotic cell rate (Fig. 1). Moreover, we found that siRNA directed against the Bcl-2 gene reduced both its mRNA and protein expression in the canine melanoma cell line MCM-N1 (Fig. 2). These results were similar results of siRNA targeting Bcl-2 in human malignant melanoma cell lines [11]. Our study points to the importance of Bcl-2 in canine melanoma for inducing apoptosis, and reinforces the notion of Bcl-2 as a putative therapeutic target in tumors. Additional experiments are warranted to understand how canine Bcl-2 controls spontaneous apoptosis in canine tumors in vivo.

ACKNOWLEDGMENTS. This study was supported by grants from Nihon University, from the Academic Frontier Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and Kariya Animal Hospital Inc.

REFERENCES


