Transferred BCR/ABL DNA from K562 Extracellular Vesicles Causes Chronic Myeloid Leukemia in Immunodeficient Mice

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Abstract

Our previous study showed that besides mRNAs and microRNAs, there are DNA fragments within extracellular vesicles (EVs). The BCR/ABL hybrid gene, involved in the pathogenesis of chronic myeloid leukemia (CML), could be transferred from K562 EVs to neutrophils and decrease their phagocytic activity in vitro. Our present study provides evidence that BCR/ABL DNAs transferred from EVs have pathophysiological significance in vivo. Two months after injection of K562 EVs into the tail vein of Sprague-Dawley (SD) rats, they showed some characteristics of CML, e.g., feebile, febrile, and thin, with splenomegaly and neutrophilia but with reduced neutrophil phagocytic activity. These findings were also observed in immunodeficient NOD/SCID mice treated with K562 EVs; BCR/ABL mRNA and protein were found in their neutrophils. The administration of actinomycin D, an inhibitor of de novo mRNA synthesis, prevented the abnormalities caused by K562 EVs in NOD/SCID mice related to CML, including neutrophilia and bone marrow hyperplasia. As a specific inhibitor of tyrosine kinases, imatinib blocked the activity of tyrosine kinases and the expression of phospho-Crk, induced by the de novo BCR/ABL protein caused by K562 EVs bearing BCR/ABL DNA. Our current study shows the pathophysiological significance of transferred tumor gene from EVs in vivo, which may represent an important mechanism for tumorigenesis, tumor progression, and metastasis.

Introduction

Extracellular vesicle (EV) is an important mode of intercellular communication, besides soluble factor and tunneling nanotube. EVs carry signals within or at their limiting membrane, providing a mechanism by which cells can exchange more complex information than previously thought [1–3]. Several studies have shown that in addition to proteins, mRNAs, and microRNAs, there are DNA fragments within EVs [4–8]. Our previous study showed the existence of DNA in EVs that could be transferred from one cell to another by endocytosis or fusion. The transferred EV DNAs have pathophysiological significance, not only to increase the DNA-coding mRNA and protein levels, but also influence the function of the recipient cells [4].

There is increasing evidence that EVs play a pivotal role in tumorigenesis, which can occur in adjacent and remote locations. EVs shed from tumor cells have the potential to increase tumor survival and expansion, independent of cell-to-cell contact. Tumor-derived EVs are fully equipped to facilitate the escape of tumor cells from immune surveillance and at the same time be involved in the establishment of an optimal environment for newly formed and metastatic tumor cells. It is interesting to find that the tumor-derived EVs could prod normal cells towards a tumor phenotype [9]. The role of the EV DNAs in this phenomenon is not clear.

As a special tumor, chronic myeloid leukemia (CML) is a clonal myeloproliferative disease, characterized by the oncogenic Philadelphia chromosome, formed by a reciprocal translocation between chromosomes 9 and 22, resulting in the novel chimeric protein BCR/ABL (breakpoint cluster region, BCR; Abelson murine leukemia viral oncogene, ABL), that dictates the pathophysiology of CML [10–12]. There are several pieces of evidence of increased quantity of malignant and invasive tumor-EVs in the body fluids that may contribute to the progression and metastasis in CML patients [9,13]. Our previous in vitro study showed that the BCR/ABL hybrid gene could be transferred from K562 EVs to neutrophils, causing a

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decrease in their phagocytic activity. Whether or not the transferred BCR/ABL DNA has pathophysiological significance in vivo is not known. Our present study provides the evidence that transferred EV BCR/ABL DNA has pathophysiological significance in vivo experiment. After injection, via tail vein, of K562 EVs into Sprague-Dawley (SD) rats or immunodeficient NOD/SCID mice for two months, the SD rats and NOD/SCID mice showed some characteristics of CML, e.g., feeble, febrile, thin, with splenomegaly and neutrophilia but with reduced neutrophil phagocytic activity. We found the BCR/ABL DNA, mRNA, and protein in the neutrophils of K562 EV-treated animals. Moreover, inhibition of de novo mRNA synthesis by actinomycin D prevented the features caused by K562 EVs in NOD/SCID mice, including characteristics of CML such as neutrophilia and bone marrow hyperplasia. As a specific inhibitor of tyrosine kinases, imatinib blocked the activity of tyrosine kinases and the expression of phospho-Crkl, induced by the de novo BCR/ABL protein caused by K562 EVs bearing BCR/ABL DNA. Our present study shows the pathophysiological significance of transferred tumor gene by EVs in vivo, which may represent an important mechanism for tumorigenesis, tumor progression, and metastasis.

Materials and Methods

1. Cell isolation and culture

K562 and HEK293 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured at 37°C in 95% air/5% CO2 atmosphere in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Life Technologies), supplemented with 10% fetal bovine serum (Gibco, Life Technologies).

2. Isolation of EVs

EVs were isolated from cell culture medium by differential centrifugation according to our previous study [4]. After removing cells and other debris by centrifugation at 300 g for 20 min, the supernatants were centrifuged again at 1,500 g for 20 min and the initial pellets were discarded. After recentrifugation at 110,000 g for 70 min, pellets containing the EVs were resuspended in phosphate-buffered saline (PBS). The isolated EVs were then subjected to DNase I (20 Kunitz units/ml EVs) (Sigma-Aldrich Co., St. Louis, MO, USA) digestion (30 min at 37°C) to remove any DNA outside the EVs. EDTA (10 mmol/L EVs) was then added to the EVs, and incubated for 5 min at 65°C to inactivate any residual DNase. These were the EVs used in the experiments.

3. DNA extraction from EVs

The isolated EVs were subjected to DNase digestion to remove the DNA exterior of the EV, as described above. Total DNA was extracted from EVs with the TIANamp Genomic DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China), following the manufacturer’s instructions. The DNA deposit was eluted by about 50 μl of sterile deionized water. The quality and quantity of extracted DNA were measured by spectrophotometry (A260 nm) and agarose gel electrophoresis.

| Primer name | Sequence (5’-3’) | Product length | Amplified gene |
|-------------|-----------------|----------------|----------------|
| BCR/ABL-gD-FF | 5’-TCCACTGACCCATGGATTAAAGCA-3’ | 418 bp | K562 BCR/ABL gDNA[10] |
| BCR/ABL-gD-RP | 5’-GGTGAATTGGAAGAAGCAGCGT-3’ | 259 bp | K562 BCR/ABL mRNA |
| BCR/ABL-cD-FF | 5’-CGGGACGACGAGAATGTG-3’ | 132 bp | Human ACTB mRNA |
| ACTB-cD-FP | 5’-CCGAGAAGATCCTCCATCTCC-3’ |
| ACTB-cD-RP | 5’-GTGATTCCTTCTGCATCATG-3’ |

Table 1. Sequence of PCR amplification primers.
Transferred DNA by EVs in Tumorigenesis

A

B

C

1 cm
4. Qualitative PCR of DNA
DNAs within EVs were amplified by qualitative PCR, using 2×Taq PCR MasterMix (Tiangen), according to the manufacturer’s instructions. Twenty-five microliters of the final reaction contained 12.5 μl of 2×Taq PCR MasterMix, 1 μl of sense primer, 1 μl of antisense primer, and 10.5 μl of DNA extract. Thermocycling was conducted using a MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, California, USA), initiated by a 5 min incubation at 94°C, followed by 40 cycles at 94°C for 30 sec, 58°C for 45 sec and 72°C for 45 sec. All primers used are listed in Table 1.

5. RNA isolation and RT-PCR of mRNA
Total RNA of EVs or cells was extracted using TRIzol Reagent (Life Technologies Co., Carlsbad, California, USA). Residual DNA was removed by DNase I digestion following RNA isolation, as described below. The mixture of 11 μl purified RNA and 1 μl of 25 pmol/μl randomized primer (Toyobo Co., Osaka, Japan) in an RNA-free microcentrifuge tube was denatured by boiling at 65°C for 5 min, followed by immediate cooling on ice. Twelve μl of denatured RNA, 4 μl of 5× RT buffer (Toyobo), 2 μl of dNTP mixture (10 mM each of dNTPs, Toyobo), 1 μl of 10 U/μl RNase inhibitor (Toyobo), and 1 μl ReverTra Ace (a reverse transcriptase) (Toyobo) were mixed into a total volume of 20 μl transcriptase reagents. The mixture was incubated at 30°C for 10 min, 42°C for 30 min, 70°C for 5 min, and 4°C for 5 min to allow for the synthesis of first-strand complimentary DNA (cDNA). Subsequently, qualitative PCR of cDNA was performed using 2×Taq PCR MasterMix (Tiangen) by the MyCycler thermal cycler (Bio-Rad), as described above.

6. Immunoblotting
The polyclonal rabbit anti-human and rat c-ABL (BCR-ABL) antibodies were purchased from Cell Signaling Technology, Inc., Danvers, MA and diluted 1:1000 [11]. Proteins were visualized using the enhanced chemiluminescence system.

7. Animal experiments
SD rats or NOD/SCID mice were maintained on a 12 hr light/dark cycle in a pathogen-free animal facility at Daping Hospital. The fresh isolated EVs were injected to rats or mice every day. At 4 weeks of age, the rats were injected with PBS (200 μl), actinomycin D (7 μg/kg), K562 EVs (2×10⁶) and actinomycin D (7 μg/kg), K562 EVs (2×10⁶) in 200 μl or K562 cells (2×10⁶) in 200 μl via the tail-vein. Rectal temperatures and body weights were recorded every week. After 2 months, the animals were sacrificed, the spleens were obtained, and spleen to body weight ratio was calculated. In addition, bone neophrophils were counted using Sysmex XE-2100 hematology analyzer (Sysmex Inc., Kobe, Japan). Bone marrow cells on a bone marrow smear with Wright’s staining were examined under a microscope using 10×40 lens. Sections of NOD/SCID mice spleens were stained with hematoxylin-eosin (HE) and examined under a microscope using 10×40 lens.

Neutrophils were isolated from rat or mouse peripheral blood with neutrophil separating medium (TBD Co. Ltd., Tianjin, China) and then washed with PBS three times to remove any contaminating EVs. For measurement of BCR/ABL levels, genomic DNA, total RNA, and cell lysates were extracted from the neutrophils, and then analyzed by PCR, RT-PCR, and immunoblotting, as previously described [4]. All experiments were approved by the Third Military Medical University Animal Use and Care Committee.

8. Assay for phagocytic activity of neutrophils
Phagocytic activity of neutrophils was detected by ink phagocytosis test. After the addition of 10 μl India ink, 100 μl of heparin anti-coagulated peripheral blood were incubated at 37°C for 4 hrs and the blood smears were washed with Wright's stain. To evaluate the level of phagocytic activity of neutrophils, ink-phagocytic cells and ink-particles within these cells were counted from 100 neutrophils on a blood smear with Wright’s stain, under a microscope using 10×100 oil lens. Phagocytic rate (the sum of ink-phagocytic cells per 100 neutrophils) and index (the sum of ink-phagocytic particles per 100 neutrophils) were calculated.

9. Activity of tyrosine kinases by ELISA
Mouse neutrophils were incubated with K562 EVs (10⁵/mL) and/or imatinib (0.5 μmol/L), a specific inhibitor of tyrosine kinases, for 24 h. About 10⁵/mL of neutrophils in 100 μL PBS were collected by centrifugation at 500×g for 20 min. Then, the cell lysates of neutrophils were prepared by freeze-thawing for three times. The supernatants samples were obtained by centrifugation at 1800×g for 30 min. Tyrosine kinases activity was determined using a protein tyrosine kinases ELISA kit (R&D Systems, Inc.) according to the manufacturer’s recommendation.

10. Expression of phospho-CrkI by immunofluorescence
The expression of phospho-CrkI in the neutrophils was detected by immunofluorescence. The polyclonal rabbit anti-mouse phospho-CrkI antibodies (Tyr207) were purchased from Cell Signaling Technology, Inc., and diluted 1:100. Goat anti-rabbit antibodies labeled with FITC (1:200) were binding to phospho-CrkI antibodies. Nuclei (DAPI-staining, blue) and...
phospho-Crk1 (FITC, green) were viewed through a Zeiss LSM 510 META laser confocal microscope (Carl Zeiss) at individual excitation wavelengths (350 nm for DAPI and 490 nm for FITC). Fluorescence intensity of phospho-Crk1 in neutrophils was determined under four visual fields.

11. Statistical analysis

The data are expressed as mean ± SD. Comparison within groups was made by ANOVA for repeated measures, and comparison among groups was made by factorial ANOVA and Duncan’s test (t-test when only 2 groups were compared). A value of P<0.05 was considered significant.

Results

1. Effect of K562 EVs on the pathophysiological changes in SD rats

Our previous study showed that that BCR/ABL hybrid gene could be transferred from K562 EVs to HEK293 cells or neutrophils [4]. To determine whether or not there is pathophysiological significance of the transferred BCR/ABL hybrid gene in EVs in vivo, we injected, via the tail vein, K562 EVs into SD rats. To decrease the immunological response due to the xenogeneic immunological incompatibility between humans and rats, we treated the SD rats with dexamethasone sodium phosphate (1.0 mg/kg/day) [15]. Two months later, SD rats, injected with K562 EVs, showed some characteristics of CML, e.g., feeble, febrile, thin, with splenomegaly and neutrophilia (Figure 1A, but with reduced neutrophil phagocytic activity (Figure 1B), similar to those seen in CML [16]. Consistent with the in vitro study, the neutrophils of the K562 EV-injected SD rats were found to express BCR/ABL protein (Figure 1C).

To determine the immune and inflammatory responses of SD rats bearing K562 cells or their EVs, we measured the ratio of CD4+ T lymphocytes to CD8+ T lymphocytes and plasma C-reactive protein (CRP) levels. We found that those above-mentioned parameters were not different among control SD rats and SD rats treated with K562 cells or K562 EVs (Figures 1D-a and 1D-b).

2. Effect of K562 EVs on the pathophysiological changes in NOD/SCID mice

Although the SD rats were treated with dexamethasone prior to administration of the K562 EVs, it would be difficult to eliminate completely the immunological reaction due to the xenogeneic immunologically incompatible systems. Therefore, we re-performed the rat experiment in the immunodeficient mouse, NOD/SCID mouse. NOD/SCID mice were injected with K562 EVs or K562 cells and/or actinomycin D, via tail-vein, every three days for 2 months. Two months later, NOD/SCID mice, injected with K562 EVs, showed characteristics of CML similar to the SD rats injected with K562 EVs, e.g., feeble, febrile, and thin, and with splenomegaly, (Figures 2A, 2B and 2C-a). The spleens were swollen and infiltrated by leukemia cells, observed by H.E. staining (Figure 2C-b). Hyperplastic bone marrow (Figure 3A) and increased neutrophils count in peripheral blood (Figure 3B) were observed in the K562 EV-injected mice.

Our published in vitro study showed that the transferred BCR/ABL DNA from K562 EVs were functional, which could be transcribed into BCR/ABL mRNA and protein that subsequently affected the phagocytic activity of neutrophils. To determine whether or not the transcription of BCR/ABL gene played a key role in the pathogenesis of CML, we treated the NOD/DCID mice with actinomycin D (7.0 µg/kg), an inhibitor of de novo mRNA synthesis. Although actinomycin D, by itself had no effect, it blocked the development of CML caused by K562 EVs (Figures 2A, 2B and 2C-a), i.e., the hyperplastic bone marrow and neutrophilia in the K562 EVs-treated mice were no longer observed (Figures 3A and 3B), indicating that there was de novo transcription of BCR/ABL mRNA, as well as protein synthesis in vivo.

3. BCR/ABL expression in neutrophils from NOD/SCID mice

As indicated in our published in vitro study, the de novo transcription of BCR/ABL DNA transferred by K562 EVs plays an important role in the pathogenesis of CML [4]. Therefore, we examined the expressions of BCR/ABL DNA, mRNA, and protein in the peripheral blood of NOD/SCID mice injected with K562 EVs or K562 cells and found them to be expressed in their neutrophils (Figure 4). Actinomycin D (7.0 µg/kg), by itself, had no effect on BCR/ABL DNA expression (Figure 4A), but it decreased the BCR/ABL mRNA and protein expressions in the neutrophils (Figures 4B and 4C) of K562 EV-injected NOD/SCID mice.

4. Inhibition of imatinib on the de novo BCR/ABL transcription

To validate whether or not the de novo BCR/ABL protein influences tyrosine kinases in the neutrophils transferred by K562 EVs bearing BCR/ABL DNA, we treated these neutrophils with imatinib (0.5 µmol/L), a specific inhibitor of tyrosine kinases. We determined the activity of tyrosine kinases in neutrophils by ELISA. Although imatinib, by itself, had no significant effect, it blocked the activity of tyrosine kinases in the neutrophils transferred by K562 EVs (Figures 5A). As Crk1 is a prominent substrate...
of BCR/ABL oncprotein in CML and binds to BCR/ABL, we detected the expression of phospho-CrkI in the neutrophils by immunofluorescence. After imatinib treatment, the higher levels of phospho-CrkI in the neutrophils transferred by K562 EVs was no longer observed (Figures 5B), indicating the de novo BCR/ABL protein was functional with tyrosine kinase activity.

**Discussion**

Cell to cell communication is involved in tissue morphogenesis, wound healing, and tumor metastases. Direct communication between mammalian cells occurs either by the transfer of information through EVs or physical connection through nanotubes [7,17,18]. Several signals via EVs can contribute to diverse cancer phenomena, such as field effect and priming of the metastatic niche [8]. For example, CML cell-derived exosomes induced angiogenic activity in endothelial cells, revealing a key role for EVs in both the pathogenesis of leukemia and its metastasis [19]. Our previous study found that the transferred BCR/ABL hybrid gene in EVs could increase the BCR/ABL mRNA and protein levels in neutrophils in vitro [4]. The present study showed that the BCR/ABL hybrid gene, the unique pathogenic gene causing CML, could be transferred from EVs in vivo, resulting in CML. These results are consistent with the view that *Plasmodium falciparum*-infected red blood cells directly communicate between parasites within a population using exosome-like vesicles that are capable of delivering genes [7].

In the present study, the initial experiment involving the injection of K562 EVs into SD rats has a limitation: a xenogeneic immunological incompatibility. Therefore, one cannot rule out that the immune response of SD rats to human antigens present in K562 leukemic cells and their EVs could have caused the CML-like symptoms, such as being feeble, febrile, thin, and with splenomegaly and neutrophilia. To overcome this limitation, we re-performed the experiment in immunodeficient NOD/SCID mice. To further confirm the transfer of EV DNA in the recipient animal, actinomycin D, an inhibitor of de novo DNA transcription, was used in the in vivo experiment. Actinomycin D blocked the development of the characteristics of CML caused by the injection of K562 EVs in NOD/SCID mice. Therefore, the injection of K562 EVs in NOD/SCID mice caused, in vivo, de novo BCR/ABL mRNA, and protein synthesis. However, actinomycin D is non-specific inhibitor for BCR/ABL transcription, thus we used another specific inhibitor of tyrosine kinases, which is activated by BCR/ABL protein in CML. Moreover, we also detected CrkI, which is a prominent substrate of the BCR/ABL oncprotein in CML. As described above, imatinib blocked the activity of tyrosine kinases and the expression of phospho-CrkI, induced by the de novo BCR/ABL protein caused by K562 EVs bearing BCR/ABL DNA, indicated that the de novo BCR/ABL protein is taken in the pathogenesis of CML.

The chimeric BCR-ABL protein is involved in the pathophysiology of CML [12]. Previous study suggest that CML EVs can freely and invasively deliver BCR/ABL gene from CML cells into normal neutrophils and then influence their function in the bone marrow. Although normal EVs may instruct the bone marrow to prevent metastatic leukemia [9], the leukemic EVs can promote the mobilization of neutrophils that may play an important role in leukemic invasion and metastasis. This may explain why most hematological malignancies, especially leukemia, progress rapidly. Unfortunately, the molecular mechanisms of EV biogenesis and secretion are still not well described, but the present results suggest that genetic changes could be involved in these phenomena. It is well known that tumor EVs contribute to the horizontal propagation of oncoproteins and genetic material [8,20–23]. However, our study shows that the transfer of the BCR/ABL gene from tumor-derived EVs to neutrophils may enhance the invasive and metastatic process in vivo.

In conclusion, we have demonstrated that cell to cell communication occurs between leukemia cells and normal neutrophils and that this provides a mechanism for increasing tumorigenesis. This is a key advantage for tumor cells in maintaining invasion for survival. This process is potentially an excellent target for therapeutic approaches to block tumor progression and prevent the spread of tumor drug resistance.
Figure 5. Inhibition of imatinib on the de novo BCR/ABL protein. (A): The activity of tyrosine kinases in the neutrophils. Neutrophils were incubated with K562 EVs (10^5/mL) and/or imatinib (0.5 μmol/L) for 24 h. The activity of tyrosine kinases in neutrophils were determined by ELISA (* P < 0.01 vs. control, n = 6). (B): Phospho-CrkI protein expression in the neutrophils. Neutrophils were incubated with K562 EVs (10^5/mL) and/or imatinib (0.5 μmol/L) for 24 h. The representative images (a) and protein expression (b) of phospho-CrkI in the neutrophils were detected by immunofluorescence (* P < 0.01 vs. other group, n = 4). Green and blue fluorescences indicate phospho-CrkI protein and Nuclei, respectively. Normal neutrophils (1), K562 EVs (2 × 10^5) (2), imatinib (0.5 μmol/L) (3), K562 EVs (2 × 10^5) and imatinib (0.5 μmol/L) (4).

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Author Contributions
Conceived and designed the experiments: JC GW CZ. Performed the experiments: JC GW XT YH CC NW XZ FZ. Analyzed the data: CL XC. Contributed reagents/materials/analysis tools: CZ DH LZ. Wrote the paper: JC CZ PAJ.

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