Alterations in Tyrosine Protein Phosphorylation Induced by Matrine in Leukemia Cells

Xiao-Shan Liu and Jikai Jiang

Center for Molecular Biology, School of Medicine, Shantou University, Shantou, Guangdong 515031, China

Abstract

Matrine is an alkaloid obtained primarily from roots of *Sophora flavescens* Ait. (Leguminosae), an herb used in traditional Chinese medicine to treat a range of disease. So far, the underlying molecular mechanism for the therapeutic effects of matrine has been poorly understood. In this work, we investigated the effects of matrine on tyrosine protein phosphorylation in human cells through immunoblotting. In bcr/abl-positive K562 cells, tyrosine phosphorylations of several proteins are altered in both time-and dose-dependent manners. Such alternations are distinct from those induced by the bcr/abl-targeting inhibitor STI571 and can be blocked by the later. The treatment of bcr/abl-negative lymphocytic and myeloid cell lines by matrine produced different patterns. In Raji cells, the tyrosine phosphorylation was the only protein affected. In U937 cells, no changes were observed. The findings in this work protein the first piece of evidence that matrine can alter the cellular signal transduction network at a molecular level. The observed changes in tyrosine phosphorylation of specific proteins provide important clues for uncovering the molecular mechanism of the therapeutic effects of matrine.

Keywords: Leukemia, matrine, tyrosine protein phosphorylation.

Introduction

*Sophora flavescens* Ait. (Leguminosae) (SF) is the dry root of a leguminous plant growing in China, Japan, and some European countries. It is commonly used in traditional Chinese medicine to treat a range of disease, including cancer (Xu et al., 1998), viral hepatitis (Long et al., 2004), cardiac arrhythmia (Li et al., 2000), and skin diseases (Zhang, 1996). Matrine (C15H24N2O) and oxy-matrine (C15H24N2O2), so far uniquely found only in *Sophora* species, are the major alkaloid components in this herb. These compounds are believed to be the main contributors to the various therapeutic activities of SF (Niu, 1997).

From clinical studies, a diverse range of physiological and pharmacological effects of matrine have been reported, including declined viral load and reduced liver damage in viral hepatitis (Zhang et al., 2001; Liu et al., 2003) and inhibited tumor growth and reinforced immune functions in cancer therapy (Ma et al., 1996). Matrine can also counteract heart arrhythmia in various contexts (Zhang et al., 1990; Ai et al., 2001). Reportedly, the same compound combined with other drugs can be effective in the treatment of a number of skin diseases (Ding et al., 2002).

Although widely appreciated, the above therapeutic effects of matrine have been so far poorly understood in terms of their underlying mechanisms. Studies at the cellular level should be able to shed light on this issue. In a previous study, we discovered that a crude mixture of SF at a dose of 12 mg/ml (w/v) leads to growth inhibition and multilineage differentiation in human erythro-leukemia K562 cell line (Qin et al., 1994). Furthermore, we found that pure matrine (with 99% purity detected by HPLC) at 0.1 mg/ml leads to proliferation inhibition and erythroid differentiation in K562 cells (Zhang et al., 2001).

K562 is a bcr/abl-positive cell line established from chronic myelogeneous leukemia (CML) in blast crisis (Lozzio et al., 1975). The bcr/abl fusion oncogene, derived from Philadelphia chromosome (Ph+)(Nowell et al., 1960), which is the hallmark of diseases including nearly all cases of chronic myelogenous leukemia.
Shanxi Province, China, and prepared as a 10 mg stock solution in sterile H2O. Matrine was purchased from Xian Botany Garden of Reagents stock solution in sterile H2O. STI571 was provided by Novartis Pharmaceuticals (Switzerland), and prepared as a 5 mM stock solution in sterile H2O.

**Materials and Methods**

**Cells**

K562, U937, and Raji cell lines were cultured separately in RPMI 1640 supplemented with penicillin, streptomycin, and 10% FCS. They were maintained in a 37°C, 5% CO2, fully humidified incubator, passed twice weekly.

**Reagents**

Matrine was purchased from Xian Botany Garden of Shanxi Province, China, and prepared as a 10 mg/ml stock solution in sterile H2O. STI571 was provided by Novartis Pharmaceuticals (Switzerland), and prepared as a 5 mM stock solution in sterile H2O.

**Experimental format**

Logarithmically growing cells were collected, the cells were diluted to final concentration of 2×10⁶ cells/ml, to which were added the designated drugs, and the cells were placed in the incubator for intervals. The final concentration for matrine was 0.1, 0.2, 0.5 mg/ml, respectively, and for STI571, 0.1, 0.25, 0.5, 1.0 μM. At the end of the incubation period, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at 1000×g for 3 min at 4°C, and prepared for analysis as described below.

**Immunoblot analysis**

A minor modification of a method described previously was used (Dorsey et al., 2000). After drug treatment, cells were pelleted by centrifugation, lysed immediately in buffer [1% Triton X-100, 150 mM NaCl, 2.5 mM, Tris-HCl (pH 7.2), 0.5 mM EDTA, 0.5 mM Na₃VO₄] supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals, Germany). Protein concentration was determined using a Micro BCA kit (Beyotime Biotechnology, China). Equal amounts of proteins (60 μg) were boiled for 5 min, separated by SDS-PAGE (5% stacker and 10% resolving), and electroblotted to a nitrocellulose membrane. After blocking in TBS-T (0.1% Tween-20) and 5% milk overnight at 4°C, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody (mouse monoclonal antibody PY20, BD Transduction Laboratories, USA) for 1 h at room temperature. Blots were washed three-times (5 min each) in TBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 1 h at room temperature. Blots were again washed three-times (5 min each) in TBS-T and then developed using a chemiluminescence assay (ECL-plus kit; Amersham, USA).

**Results**

Matrine has been proven active in regulating gene expression and cell fates in mammalian cells (Tang et al., 2002; Feng et al., 2003). In summary, the following results show that matrine can alter tyrosine protein phosphorylation and that its effects are cell-type dependent. In the bcr/abl-positive K562 cells, matrine causes both up-(proteins migrating at 220 [P220], 130 [P130], 90 [P90] and 69 [P69] kDa and down-(36 [P36] and 34 [P34] kDa) regulations. In the bcr/abl-negative Raji cells, matrine has no obvious effect for the down regulation of a protein at 36 kDa. In another bcr/abl-negative U937 cell line, matrine has no observable effects.

**Effects of matrine and STI571 on tyrosine protein phosphorylation in K562 cells**

Figure 1 shows a time-course analysis of K562 cells exposed to 0.1 mg/ml matrine. This figure reveals increases in tyrosine phosphorylation of proteins migrating at 220 (P220), 130 (P130), 90 (P90), and 69 (P69) kDa at 30 min after matrine addition and lasting for the entire experiment period. In the same cells, phosphorylations of two proteins at 36 and 34 kDa were initially down-regulated, then recovered to their corresponding control levels by 24 h. Figure 2 shows a dose-response study at 12 h. It revealed that in cells treated by higher...
concentrations of matrine, the upregulation of the above proteins declined somewhat, while the downregulation of protein at 36 and 34 kDa remained unchanged.

Figure 3 shows that results for K562 cells exposed to STI571 alone for 12 h. The same proteins affected by matrine showed a decrease in their tyrosine phosphorylation. The addition of matrine did not seem to affect the changes induced by STI571 alone. Alternations similar to that shown in Figure 3 were observed in K562 cells treated by both 0.1 mg/ml matrine and 1 μM STI571 for 12 h (data not shown).

Effects of matrine and STI571 on tyrosine protein phosphorylation in other leukemia cells

Compared to bcr/abl-positive K562 cells, bands and signals in immunobots detecting tyrosine phosphorylation of proteins from total cell lysis were significantly weaker in bcr/abl-negative Raji or U937 cells. The chimeric bcr/abl gene encodes the fusion protein P210 with constitutive tyrosine kinase activity. This aberrant bcr/abl kinase activity is the key factor contributing to the abnormal biological activity of bcr/abl-positive cells (Lugo et al., 1990).

In Raji cells treated by 0.1 mg/ml of matrine, tyrosine phosphorylation of protein with 36 kDa was diminished dramatically at 30 min and almost disappeared at 12 h, but elevated to the control level by 24 h. The striking decline in phosphorylation of this protein was also found in Raji cells treated with 0.2 or 0.5 mg/ml matrine for 12 h (Fig. 4). No other obvious effects were observed.

In the case of U937 cells treated by matrine, no obvious effects was observed (data not shown). STI571 had no observable effects on either Raji or U937 cells (data not shown).

Discussion

In a previous study, we showed that matrine induces erythroid differentiation of partial cells in K562 cell line (Zhang et al., 2001). The current results show that at the molecular level, matrine can alter protein tyrosine phosphorylation in these cells. However, the mechanisms of erythroid differentiation in the K562 cell line are still unclear. One reason is that while the downstream signals engaged by bcr/abl expression are common to a number of oncogenes (Ras, MAPK, PI3K, AKt) (Puil et al., 1994; Shorski et al., 1995, 1997; Cortez et al., 1997), the reported signal pathways involved in the erythroid
differentiation of K562 cells induced by different chemical compounds may be distinct. For example, the hemin-induced erythroid differentiation pathway has been reported to be accompanied by reduction in levels of tyrosine-phosphorylated proteins (Richardson et al., 1987) while, to the contrary, butyrate induces both dose and time-dependent tyrosine phosphorylation of several proteins, which can be blocked by the tyrosine kinase inhibitor genistein (Rivero et al., 1996). The designed small molecule STI571, and inhibitor of the \textit{bcr}/abl protein, can also induce K562 cells toward erythroid differentiation as well as significant apoptosis (Fang et al., 2000; Jacquel et al., 2003). This pathway involves the growth factor-cytokine signaling cascades, which are also involved in normal hematopoiesis (Laneuville et al., 1991; Sattler et al., 1997). Given our current understanding of the erythroid differential process, we cannot yet provide a more detailed picture for possible connection between erythroid differentiation and changes in tyrosine phosphorylation in matrine-treated K562 cells. The data presented here strongly suggest such connections. An important future step will be to identify the exact altered proteins shown in this work.

We observed that STI571 reduces the tyrosine phosphorylation of most of the proteins examined in our experiments in K562 cells. The same compounds has no effect on Raji or U937 cells. This is consistent with the known fact that STI571, a proven effective medicine in CML therapy, acts as a specific inhibitor targeting \textit{bcr}/abl. Our dose-response experiments show that the minimum required concentration of STI571 for the above effects is around 0.25 \( \mu \)M, similar to the reported concentration required for effective inhibition of the \textit{bcr}/abl kinase activity \textit{in vitro} (Druker et al., 1996). This further indicates that at the molecular level, the observed effects of STI571 on K562 cells is through the direct inhibition of the kinase activity of \textit{bcr}/abl.

Although both matrine and STI571 induce alterations in tyrosine phosphorylation in \textit{bcr}/abl-positive K562 cells, their effects are quite distinct. Matrine upregulates tyrosine phosphorylation of some proteins while downregulating some other proteins. Only downregulation occurs after the treatment by STI571. The counterpart for this molecular level difference at the cellular level is the distinction between the cellular effects of matrine and STI571: unlike STI571, no apoptosis was observed in matrine-treated K562 cells. The difference also implies that \textit{bcr}/abl kinase is not the direct molecular target of matrine.

The observed interferences between the effects of STI571 and matrine may lead to insights into their possible mechanisms. Assuming that the kinase activity of \textit{bcr}/abl is the sole direct target of STI571, several possible roles of the \textit{bcr}/abl protein can be hypothesized to explain the observed changes in tyrosine phosphorylation caused by matrine. First matrine may upregulate the level of \textit{bcr}/abl through some unknown network of interactions, which in turn causes the observed upregulation. Second, \textit{bcr}/abl may be required to activate some
unknown module in the overall network, which in turn is required for the effects of matrine. Alternatively, matrine may up- or downregulate the levels of proteins that act as substrates of bcr/abl. In all cases, STI571 would block the effects of matrine, as shown in our experiments. As STI571 also blocks the downregulation effects of matrine, the first hypothesis seems unlikely, unless we assume that the hypothetical higher level of bcr/abl may cause the observed downregulations as well. Nevertheless, matrine does perturb the tyrosine phosphorylated proteins, which are of crucial importance in cellular network regulations.

The effects of matrine on the bcr/abl-negative Raji and U937 cells appears to indicate that the upregulation of protein tyrosine phosphorylation requires bcr/abl, consistent with the interferences between STI571 and matrine observed in K562 cells.

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References


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