Metformin Selectively Targets Cancer Stem Cells, and Acts Together with Chemotherapy to Block Tumor Growth and Prolong Remission

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Abstract

The cancer stem cell hypothesis suggests that, unlike most cancer cells within a tumor, cancer stem cells resist chemotherapeutic drugs and can regenerate the various cell types in the tumor, thereby causing relapse of the disease. Thus, drugs that selectively target cancer stem cells offer great promise for cancer treatment, particularly in combination with chemotherapy. Here, we show that low doses of metformin, a standard drug for diabetes, inhibits cellular transformation and selectively kills cancer stem cells in four genetically different types of breast cancer. The combination of metformin and a well-defined chemotherapeutic agent, doxorubicin, kills both cancer stem cells and non–stem cancer cells in culture. Furthermore, this combinatorial therapy reduces tumor mass and prevents relapse much more effectively than either drug alone in a xenograft mouse model. Mice seem to remain tumor-free for at least 2 months after combinatorial therapy with metformin and doxorubicin is ended. These results provide further evidence supporting the cancer stem cell hypothesis, and they provide a rationale and experimental basis for using the combination of metformin and chemotherapeutic drugs to improve treatment of patients with breast (and possibly other) cancers. [Cancer Res 2009;69(19):7507–11]

Introduction

Chemotherapeutic treatments for cancer can effectively reduce tumor mass, but the disease often relapses. To explain this phenomenon, the cancer stem cell hypothesis suggests that tumors contain a small number of tumor-forming, self-renewing, cancer stem cells within a population of nontumor-forming cancer cells (1, 2). Unlike most cells within the tumor, cancer stem cells are resistant to well-defined chemotherapy, and after treatment, they can regenerate all the cell types in the tumor through their stem cell–like behavior. For this reason, drugs that selectively target cancer stem cells offer great promise for cancer treatment, although none are known at present. Epidemiologic studies indicate that diabetes is correlated with increased risk of breast and other cancers (3, 4), and we recently defined a transcriptional signature and drug sensitivity profile of cellular transformation linking multiple types of cancer with diabetes and other metabolic diseases. Metformin is an extensively used and well-tolerated drug for treating individuals with type 2 diabetes, obesity, and polycystic ovarian syndrome. Diabetics treated with metformin have reduced cancer risk (5, 6), although it is unclear whether metformin affects cancer directly or indirectly by inhibiting the diabetic state. Metformin inhibits the growth of breast cancer cell lines, although it also affects nontransformed cells at the concentrations tested (7–9). In nude mice, metformin modestly inhibits tumor growth of xenografts of a triple-negative breast cancer cell line that lacks the estrogen, progesterone, and HER2 receptors (8). These observations suggest the possibility that metformin might be useful as an anticancer drug in nondiabetic contexts (10, 11).

Here, we show that metformin selectively kills cancer stem cells in four genetically different types of breast cancer. The combination of metformin and doxorubicin, a well-defined chemotherapeutic drug, kills both cancer stem cells and non–stem cancer cells in culture, and reduces tumor mass and prolongs remission much more effectively than either drug alone in a xenograft mouse model. These observations constitute independent support for the cancer stem cell hypothesis, and they provide a rationale for why the combination of metformin and chemotherapeutic drugs might improve treatment of patients with breast (and possibly other) cancers.

Materials and Methods

Cell lines. MCF10A cells are mammary epithelial cells derived from fibrocystic breast tissue that was obtained from a mastectomy of a 36-y-old woman with no family history of breast cancer and no evidence of disease (12). Genetic analysis did not reveal any amplification of HER2/neu oncogene or mutations in H-Ras oncogenes, and these cells do not express estrogen receptor (ER). The experiments here use a derivative of MCF10A containing an integrated fusion of the v-Src oncoprotein with the ligand-binding domain of ER. MCF7 cells are mammary adenocarcinoma cells that express very high levels of ER, are negative for HER2/neu, and do not have strong anchorage-independent properties (13). SKBR3 cells are mammary adenocarcinoma cells that overexpress the HER2/neu receptor, have anchorage-independent properties, and form tumors in xenografts (14). MDA-MB-468 cells are derived from a triple-negative breast carcinoma that shows many of the recurrent basal-like molecular abnormalities including ER-PR-HER2–negative status, p53 deficiency, epidermal growth factor (EGF) receptor overexpression, PTEN loss and constitutive activation of the MAP/extracellular signal-regulated kinase (ERK) kinase/ERK pathway (15). MDA-MB-468 cells are very aggressive and form large tumors in xenograft experiments that resist treatment with tamoxifen or herceptin.

Cell culture. MCF-7, SKBR3, and MDA-MB-468 cells were grown in DMEM media (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), and penicillin/streptomycin (Invitrogen) at 37°C with 5% CO₂. MCF10A ER-Src cells were cultured as described previously (16) and induced to transform with 1 µmol/L 4OH-tamoxifen dissolved (Sigma) in ethanol. Morphologic changes, phenotypic transformation, and foci formation

Note: I.A. Hirsch and D. Iliopoulos made equal contributions to this work.

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occurred 24 to 36 h after tamoxifen addition, and were monitored by phase-contrast microscopy. Metformin (Sigma) dissolved in water was typically added to 0.1 mmol/L unless otherwise indicated.

Wound-healing motility assay. Cells were seeded onto six-well dishes at 1 x 10^5 per well. A single scratch wound was created using a p10 micropipette tip in to confluent cells. Cells were washed thrice with PBS to remove cell debris, supplemented with assay medium, and monitored. Images were captured by phase-contrast microscopy at 0 and 12 h after wounding.

Colony formation assay. Triplicate samples of 5 x 10^4 cells from MCF10A ER-Src were mixed 4:1 (v/v) with 2.0% agarose in MCF-10A growth medium for a final concentration of 0.4% agarose. The cell mixture was plated on top of a solidified layer of 0.5% agarose in growth medium. Cells were fed every 6 to 7 d with growth medium containing 0.4% agarose. The number of colonies was counted after 15 d.

Mammosphere culture. Mammospheres were cultured in suspension (1,000 cells/mL) in serum-free DMEM/F12 media, supplemented with B27 (1:50; Invitrogen), 0.4% bovine serum albumin, 20 ng/mL EGF (Preprotech) and 4 μg/mL insulin (Sigma) as described previously (17). Mammosphere formation was tested by placing transformed cell populations in the presence of absence of metformin under these conditions, whereas mammosphere growth was examined by adding metformin to 6-d-old mammospheres and counting the number of mammospheres 2 and 4 d after treatment.

Isolation and analysis of cancer stem cells. Flow cytometric cell sorting of transformed cell populations was performed on single-cell suspensions. Cells were stained with CD44 antibody (FITC conjugated; 555478, BD Biosciences) and with CD24 antibody (phycoerythrin conjugated; 555428, BD Biosciences). Cancer stem cells (CD44^high/CD24^low) and no-stem–transformed cells (CD44^low/CD24^high) from MCF10A ER-Src (tamoxifen treated) and MCF7, SKBR3, and MDA-MD-486 cells were treated with 0.1 mmol/L metformin, and cell growth was assessed in different time points (12, 24, 48 h). The experiments were performed in triplicate, and the data represent mean ± SD.

Tumor growth and relapse in xenografts. MCF10A ER-Src cells (5 x 10^6) were injected into the right flank of 16 female nu/nu mice (Charles River Laboratories), all of which developed tumors in 10 d with size of ~50 mm^3. The mice were randomly distributed into four groups that were untreated, or treated by i.p. injections every 5 d (three cycles) with 4 mg/kg doxorubicin, 100 μg/mL metformin, or the combination. Tumor volume (mean values and 95% confidence intervals) was measured at various times after the initial injection. All the mouse experiments were performed in accordance with Institutional Animal Care and Use Committee procedures and guidelines.

Figure 1. Metformin prevents transformation of MCF10A-ER-Src cells. A, number of cells grown in the presence or absence of 1 μmol/L 4-hydroxy tamoxifen (TAM) with the indicated concentrations of metformin for 24 h. B, phase-contrast images of cells grown in the presence or absence of 0.1 mmol/L metformin and/or tamoxifen for 36 h. C, wound-healing/invasion response assay of cells grown in the presence or absence of 0.1 mmol/L metformin and/or tamoxifen. D, relative number of foci, colonies in soft agar, and mammospheres in untreated or tamoxifen-treated cells in the presence of the indicated concentration of metformin.

Figure 2. Metformin inhibits growth of mammospheres. Six-day-old mammospheres from the indicated cell lines were or were not treated with 0.1 mmol/L metformin for 48 h, and the number of mammospheres was counted.
Results and Discussion

To examine the anticancer properties of metformin, we first used an inducible transformation model consisting of nontransformed human mammary epithelial cells (MCF-10A) containing ER-Src, a fusion of the v-Src oncoprotein with the ligand-binding domain of ER. When these cells are treated with tamoxifen, they become transformed within 24 to 36 h. The transformed cell population contains 10% cancer stem cells, as defined by expression of the CD44 marker and the ability to form mammospheres, multicellular “microtumors” that are generated in nonadherent and nondifferentiating conditions (18). In addition, we analyzed three other mammary adenocarcinoma cell lines derived from genetically and phenotypically different tumors that are treated with different drugs: ER-positive MCF7 (13), HER-positive SKBR3 (14), and triple-negative MDA-MB-468 (15). These cell lines also contain a minority population of cancer stem cells capable of mammosphere formation. In all experiments, metformin was used at a concentration that does not affect the growth of nontransformed cells.

Figure 3. Metformin (met) selectively kills cancer stem cells and functions synergistically with doxorubicin (dox). A, number of cancer stem cells (CD44\textsuperscript{high}/CD24\textsuperscript{low}; black) and cancer cells (CD44\textsuperscript{low}/CD24\textsuperscript{high}; gray) in the transformed (36 h tamoxifen treatment) MCF-10A population that was treated with doxorubicin, 0.1 mmol/L metformin, or both (n = 3). B, cancer stem cells (SC) and non–stem cancer cells (NSC) obtained by sorting were treated with 0.1 mmol/L metformin for 0, 24, and 48 h. C, tumor volume in nude mice at the indicated number of days after injection of MCF10A-ER-Src cancer stem cells that were or were not treated with 0.1 mmol/L metformin for 1 h before injection.
(0.1 or 0.3 mmol/L; Fig. 1A). Previous experiments on cancer cell
lines (7–9) used much higher concentrations of metformin
typically 10–30 mmol/L), conditions that are also toxic for
nontransformed cells.

In the inducible MCF-10A model, metformin strongly inhibits
morphologic transformation (Fig. 1B), invasive growth in wound-
healing assays (Fig. 1C), focus formation, formation of colonies in
soft agar, and generation of mammospheres (Fig. 1D). Furthermore,
metformin treatment of mammospheres derived from all four
breast cancer cell lines causes a dramatic reduction in the number
of mammospheres within 48 hours (Fig. 2) as a consequence of cell
death. As mammospheres are composed primarily of cancer stem
cells (18), this latter observation suggests that metformin may kill
cancer stem cells.

Strikingly, metformin preferentially kills cancer stem cells
(CD44<sup>high</sup>/CD24<sup>low</sup>) within a population of transformed MCF-10A
or MCF-7 cells (Fig. 3A). Similarly, when all four cancer cell lines
are sorted, cancer stem cells are quite susceptible to metformin,
whereas the standard cancer population remains essentially
unaffected (Fig. 3A). Furthermore, treatment of MCF-10A cancer
stem cells with metformin for just 1 hour blocks the ability of these
cells to form tumors in nude mice, although the drug is not present
for the month after injection (Fig. 3C). The ability of metformin to
selectively kill cancer stem cells is in marked contrast to
doxorubicin, a chemotherapeutic agent that kills cancer cells, but
not cancer stem cells. As expected from their distinct properties,
metformin works together with doxorubicin to reduce both
nonstem cancer cells and cancer stem cells in the mixed
transformed population (Fig. 3A).

In accord with the above results in cell lines, the synergy
between metformin and doxorubicin is observed upon treatment of
tumors that arise 10 days after injection of MCF-10A-ER-Src cells
into nude mice. After 15 days of treatment (three cycles every
5 days), this drug combination virtually eliminates tumors, whereas
doxorubicin alone causes only a 2-fold decrease in tumor volume
and metformin alone has little effect (Fig. 4A). Doxorubicin-treated
mice show a further reduction in tumor volume after an additional
10 days (day 35). The minimal effect of metformin alone is in
contrast to more significant effects seen in an independent report
(8), but there are many differences in experimental protocol
between these studies.

To determine the basis for why the combination of metformin
and doxorubicin is more effective than doxorubicin alone, we
examined the population of cells recovered from tumors after
three cycles of treatment (day 25). In accord with our results in
cell lines, cancer stem cells are virtually absent from mice
treated with the drug combination, whereas they are easily
detected in tumors from mice treated with doxorubicin alone
(Fig. 4B). Thus, the therapeutic advantage of metformin in the
context of conventional chemotherapy is linked to its ability to
kill cancer stem cells.

The cancer stem cell hypothesis for the progression of human
disease is based on the differential tumor-forming properties and
responses to well-defined chemotherapy of cancer stem cells and
non–stem cancer cells. A prediction of this model, heretofore
untested, is that drugs that selectively inhibit cancer stem cells
should function synergistically with chemotherapeutic drugs to
delay relapse. Strikingly, mice treated with the combination of
metformin and doxorubicin remain in remission for at least 60 days
after treatment is ended (Fig. 4A). In contrast, tumor growth
resumes 20 days after mice are treated with doxorubicin alone, and

Figure 4. Metformin and doxorubicin act in combination to reduce tumor
mass and prolong remission in nude mice. A. tumor volume (mean values and
95% confidence intervals) of mice injected with transformed MCF10A-ER-Src
cells (time 0 indicates the time of injection) that were untreated, or treated by
i.p. injections every 5 d (three cycles; arrows, the day or injections) with 4 mg/kg
doxorubicin (dox), 100 μg/mL metformin (met), or both. B, number of cancer
stem cells (CD44<sup>high</sup>/CD24<sup>low</sup>) in cells obtained from tumors treated with
doxorubicin or the combination of doxorubicin + metformin after three cycles
of treatment (day 25).
epidemiologic observation that diabetics treated with metformin have a lower incidence of cancer (5, 6). As a cancer preventative, metformin would be required on a long-term basis, and in this regard, the concentration of metformin needed for the anticancer effects observed here is considerably below that used for the treatment of diabetes. Lastly, the selectivity of metformin and doxorubicin for distinct types of cells in the tumor can explain the striking combinatorial effects on reducing tumor mass and prolonging remission in nude mice, and it provides the rationale for combining metformin with chemotherapy as a new treatment for breast (and possibly other) cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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K. Struhl dedicates this article to the memory of Joseph Struhl.

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