Regulation of Multidrug Resistance in Cancer Cells by Hyaluronan*

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Multidrug resistance in cancer cells is often due to ATP-dependent efflux pumps, but is also linked to alterations in cell survival and apoptotic signaling pathways. We have found previously that perturbation of hyaluronan-tumor cell interaction by treatment with hyaluronan oligosaccharides suppresses the phosphoinositide 3-kinase/Akt cell survival signaling pathway in cancer cells and reduces tumor growth in vivo. Here we find that these oligomers suppress both the MAP kinase and phosphoinositide 3-kinase pathways in multidrug resistant tumor cells and sensitize these cells to a variety of chemotherapeutic drugs. On the other hand, increased hyaluronan production induces resistance in drug-sensitive tumor cells. Likewise, increased expression of emmprin, which is a glycoprotein that is present on the surface of most malignant cancer cells and that stimulates hyaluronan production, also induces increased resistance. Thus, perturbation of hyaluronan signaling may provide a dual therapeutic role, since it has intrinsic suppressive effects on tumor growth as well as sensitizing cancer cells to chemotherapeutic agents.

Multidrug resistance in cancer arises by several mechanisms, among which are activated repair and detoxifying systems, restricted access to or uptake of drugs due to cell adhesion barriers, or enhanced drug efflux via broad specificity, ATP-dependent pumps (1). “Classical” multidrug resistance is usually due to enhanced drug export by ATP-dependent efflux pumps in the mdr, mrp, and related ABC transporter families. However, it has become increasingly apparent that alterations in cell survival and apoptotic signaling pathways are interconnected at many levels with multidrug resistance mechanisms in cancer cells and that drug resistance in patients may in some cases be overcome by therapeutic interventions that induce downstream events in apoptotic cascades (2–4).

Hyaluronan is a very large, linear glycosaminoglycan composed of repeating disaccharides of glucuronic acid and N-acetylgalactosamine. Although hyaluronan is distributed ubiquitously in vertebrate tissues, both in the embryo and in the adult, its organization with respect to cells is variable. In adult tissues such as the vitreous, synovial fluid and the dermis, it clearly plays an extracellular, structural role based on its unique hydrodynamic properties. However, during dynamic cellular events such as inflammation, wound repair, and tissue development, hyaluronan also interacts with cells and influences their behavior in a variety of ways (5, 6). In this context hyaluronan binds cell surface receptors such as CD44 and RHAMM that transduce intracellular signals, thus influencing cellular form and function directly and instructively (6). In a recent study, we found that perturbation of hyaluronan-cell interactions in malignant cancer cells by treatment with hyaluronan oligosaccharides (~1200–4000 Da) induces apoptosis under anchorage-independent conditions and reduces tumor growth in vivo (7). Hyaluronan oligomers compete for endogenous polymeric hyaluronan, thus replacing high affinity, multivalent and cooperative interactions with low affinity, low valency receptor interactions (8, 9). We found that these oligomers suppress the PI 3-kinase/Akt cell survival pathway, leading to proapoptotic events such as decreased phosphorylation of BAD and FKHR, increased PTEN expression, and increased caspase-3 activity (7). Current evidence indicates that these effects are mainly due to disruption of hyaluronan-CD44 interactions (7), although it remains possible that interactions with other extracellular, cell surface or intracellular binding proteins are also involved. Nevertheless, since multidrug resistance is often dependent on cell survival signaling pathways, and since hyaluronan oligomers suppress at least part of these pathways, we postulated that hyaluronan oligomers might reverse drug resistance.

Experimental Procedures

Cell Culture—MCF-7/Adr drug-resistant human mammary carcinoma cells (obtained from Dr. K. Cowan, University of Nebraska) were grown in culture for 24 h in 24-well plates in RPMI 1640 medium containing Glutamax 1 plus 10% fetal bovine serum at 37° in 5% CO2. Various concentrations of chemotherapeutic agents were then added and the cells incubated for another 24 h in the presence or absence of 100 μg/ml hyaluronan oligomers. The oligomers were a highly purified, mixed population of three to eight repeating disaccharides in length (7). Cells were harvested and the number of viable cells counted in a Coulter Counter.

Recombinant Adenovirus Infection—MCF-7 cells were infected overnight with a recombinant murine Has2 adenovirus, human emmprin adenovirus, or control β-galactosidase adenovirus, which were constructed and used as described previously (10, 11). After changing the medium, the cells were treated as described in the text and legends.

Assay of Hyaluronan—After infection with recombinant adenoviruses as above, the cells were washed and then incubated for a further 48 h. Hyaluronan was measured in the medium and cell layer by an enzyme-linked immunosorbent assay-like method (12).

Results and Discussion

We examined the effect of co-treatment with hyaluronan oligomers on drug resistance in an established system, i.e. MCF-7/Adr human mammary carcinoma cells that have been

* The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase; BAD136, serine residue 136 of BAD; BAD112, serine residue 112 of BAD; FAK, focal adhesion kinase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MAP, mitogen-activated protein.

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selected for resistance to doxorubicin (13). First, we compared the effect of hyaluronan oligomers on resistance to doxorubicin in the MCF-7/Adr cells versus the relatively drug-sensitive, parental MCF-7 cell line. We found that 100 μg/ml hyaluronan oligomers caused ∼55-fold sensitization of the MCF-7/Adr cells to the drug, but had little effect on the already sensitive MCF-7 cells (Fig. 1, A and B; Table I). We tested a range of concentrations of the oligomers and found that, whereas concentrations up to 250 μg/ml have little or no effect on cell survival when used alone, concentrations of 10 μg/ml or more have a highly significant effect on doxorubicin resistance (data not shown).

We then tested the effect of hyaluronan oligomers on resistance of these cells to other drugs, i.e. whether they affect multidrug resistance. We found that the oligomers decreased resistance to taxol by ∼12-fold, to 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) by ∼78-fold and to vincristine by ∼10-fold (Table I). Again, the oligomers had little effect on the parental MCF-7 cells (Table I). In the case of taxol there was only 2–3-fold decrease in resistance between the two cell types, yet only the MCF-7/Adr cells were affected significantly by the oligomers. It is not yet clear why the MCF-7 cells were not sensitized by the oligomers but this may be related to their endogenous levels of cell survival pathway activity. We also tested the hyaluronan oligomers in a different cell system, i.e. resistance of MDA-MB231 human mammary carcinoma cells to the folate analog, methotrexate (14). We found that these cells were 10-fold more resistant to doxorubicin treatment than controls (Fig. 2C). The effect of emmprin was reversed by treatment with hyaluronan oligomers, confirming that emmprin increases drug resistance via hyaluronan (Fig. 2D).

Recently we found that emmprin, a member of the Ig superfamily that is enriched on the surface of most malignant cancer cells (16) and that promotes tumor progression (17), also regulates hyaluronan production in tumor cells. Thus we infected MCF-7 cells with a recombinant emmprin adenovirus and found that these cells were 10-fold more resistant to doxorubicin treatment than controls (Fig. 2C). The effect of emmprin was reversed by treatment with hyaluronan oligomers, confirming that emmprin increases drug resistance via hyaluronan (Fig. 2D).

In our previous work, we have found that hyaluronan oligomers suppress the PT 3-kinase/Akt cell survival pathway. However these studies were performed in different cells than those used here, i.e. HCT116 human colon carcinoma and TA3/St mouse mammary carcinoma cells (7). Thus we tested the effects of the hyaluronan oligomers on this pathway using three independent experiments performed in triplicate.

![Fig. 1](image.png)

**FIG. 1.** Treatment with hyaluronan oligomers sensitizes drug-resistant carcinoma cells to chemotherapeutic drugs. A, MCF-7/Adr drug-resistant human mammary carcinoma cells were grown in the presence of various concentrations of doxorubicin (Doxo) with or without 100 μg/ml hyaluronan oligomers (o-HA), then cell numbers were measured. B, MCF-7 human mammary carcinoma cells, which are much more sensitive to drug treatment than the MCF-7/Adr cells (13), were treated as described in the legend to A. C, MDA-MB231 human mammary carcinoma cells were treated with various concentrations of methotrexate in the presence or absence of 100 μg/ml hyaluronan oligomers. The results in A–C are expressed as the means (±S.D.) of cell numbers from three independent experiments performed in triplicate.

**Table I**

|                  | MCF-7/Adr | MCF-7/Adr + o-HA | MCF-7 | MCF-7 + o-HA |
|------------------|-----------|------------------|-------|--------------|
| Doxorubicin      | 2.20      | 0.04             | 0.03  | 0.04         |
| BCNU             | 14.0      | 0.18             | 0.50  | 1.20         |
| Taxol            | 0.70      | 0.06             | 0.25  | 0.25         |
| Vincristine      | 0.20      | 0.02             | 0.03  | 0.03         |
| MDA-MB231 MB231 | 0.80      | 0.006            | 0.02  | 0.02         |

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MCF-7/Adr cells. As expected, we found that hyaluronan oligomers suppress phosphorylation of Akt and stimulate expression of PTEN in the presence of the various drugs, i.e. doxorubicin, taxol, vincristine (Fig. 3A), and BCNU (not shown). PI 3-kinase activity was also inhibited but there were no effects on total levels of Akt (not shown). These effects would be expected to lead to decreased phosphorylation of BAD at serine residue 136 (BADD136), the site of Akt-mediated phosphorylation. However, in MCF-7/Adr cells, as opposed to the cells used previously, we found very little phosphorylation of BAD136 in the presence or absence of the drugs or oligomers (not shown). Thus we examined the MAP kinase pathway, which also leads to BAD phosphorylation, in this case at serine 112 by Erk (18–20). We found strong phosphorylation of BAD112 in the MCF-7/Adr cells treated with the various drugs, implying that the MAP kinase pathway is more involved in phosphorylation of BAD than the PI 3-kinase pathway in these cells. We also found that BAD112 phosphorylation is inhibited by the hyaluronan oligomers (Fig. 3, A and B). In addition we found that the oligomers inhibit phosphorylation, but not total levels, of upstream components of this pathway, i.e. Erk (Fig. 3, A and B) and Raf-1 (not shown), in the presence of the various drugs. Since these experiments were done under anchorage-dependent culture conditions, we would expect the MAP kinase pathway to be activated by FAK (21, 22). Therefore we examined FAK phosphorylation in drug-treated MCF-7/Adr cells in the presence and absence of hyaluronan oligomers and found that the oligomers inhibit phosphorylation of FAK (Fig. 3A). Inhibition of p-Erk, p-BAD112, and p-FAK levels in these experiments varied from 50–90% depending on drug and oligomer dosage. Experiments were also performed with 50–1000 nM BCNU, and similar inhibition was observed (not shown).

Since increased hyaluronan production causes enhanced drug resistance in MCF-7 cells, we also determined whether these pathways were stimulated in recombinant Has2 adenovirus-infected cells. As expected, we found that phosphorylation of Akt, Erk, BAD112, and FAK was increased in drug-treated, Has2 adenovirus-infected cells compared with controls (Fig. 3C), whereas PTEN expression was decreased (not shown). Similar results were obtained with emmprin adenovirus-infected cells.

The experiments described above indicate that endogenous hyaluronan-tumor cell interactions are a crucial component of the regulation of multidrug resistance in cancer cells and that the most likely mechanism whereby hyaluronan acts is by stimulating the PI 3-kinase and MAP kinase cell survival pathways, leading to various anti-apoptotic consequences such as phosphorylation of BAD. Active, non-phosphorylated BAD interacts with prosurvival Bcl-2 family members and induces apoptosis (23). BAD is inactivated by phosphorylation at serine 136 by Akt or at serine 112 by Erk, either of which leads to anti-apoptotic consequences that can result in increased drug resistance in tumor cells (18–20). In the MCF-7/Adr human mammary carcinoma cells used here, regulation of BAD phosphorylation is mediated mainly by Erk. However, in HCT116 human colon carcinoma and TA3/St mouse mammary carcinoma cells, phosphorylation of BAD by Akt is prominent (7). In either case, treatment of the cells with hyaluronan oligomers is inhibitory.

The idea that hyaluronan-cell interactions may be related to drug resistance is also supported by past data showing that hyaluronidase enhances the action of various chemotherapeutic agents, especially when used locally (24). Of particular interest is the observation that dispersion of multicellular spheroids of EMT-6 mammary tumor cells with hyaluronidase reverses MDR1-based multidrug resistance (25, 26). The mechanistic effect of hyaluronidase in these systems is not understood but has been explained in terms of decreased cell adhesion (25) or increased drug penetration (24, 27), rather than hyaluronan-specific effects on cell survival signaling. In earlier work we showed that calcium-independent aggregation of transformed cells is due to hyaluronan-mediated, multivalent cross-bridging of receptors on adjacent cells (5, 28). Thus it is probable that hyaluronan-induced promotion of the PI 3-kinase/Akt or MAP kinase cell survival pathways is responsible for enhanced drug resistance in these spheroids, in addition to or instead of restricted drug access. Recent work shows that enhanced integrin signaling can also induce drug resistance (29). We show here that perturbation of endogenous hyaluronan-induced signaling inhibits FAK phosphorylation, a central event in integrin signaling (21, 22). Also, we have shown that hyaluronan signaling is critical for anchorage-independent

**Fig. 2.** Increased expression of hyaluronan induces drug resistance in carcinoma cells. A, untreated MCF-7 cells or MCF-7 cells infected overnight with a recombinant Has2 adenovirus or a control β-galactosidase adenovirus (β-gal) were washed, then treated in culture for 48 h with 1–1000 nM doxorubicin, followed by analysis of cell number. IC50 values: uninfected, 18 nM; β-galactosidase, 20 nM; Has2, 210 nM. B, recombinant Has2 adenovirus-infected MCF-7 cells were treated in the presence or absence of 100 μg/ml hyaluronan oligomers (o-HA) continuously during infection and subsequent treatment with doxorubicin as described in the legend to A. C, untreated MCF-7 cells or MCF-7 cells infected overnight with a recombinant emmprin adenovirus or control β-galactosidase adenovirus were treated as described in the legend to A. D, recombinant emmprin-adenovirus-infected MCF-7 cells were treated in the presence or absence of 100 μg/ml hyaluronan oligomers (o-HA) as described in the legend to B. Results are expressed as means (±S.D.) of three measurements.
growth (7), a phenomenon that has previously been related to altered integrin signaling (21, 22, 30). These observations suggest that hyaluronan-mediated signaling and integrin signaling play overlapping or interacting roles in these events.

Our results not only document a role for hyaluronan in multidrug resistance, they also indicate that perturbation of hyaluronan interactions sensitizes resistant cells. Thus, such perturbations may provide a dual therapeutic role, since they have an intrinsic effect on tumor growth and metastasis (31, 32), as well as sensitizing cancer cells to chemotherapeutic agents as shown herein.

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REFERENCES

1. Gottesman, M. M., Fojo, T., and Bates, S. E. (2002) Nat. Rev. Cancer 2, 48–58
2. Lowe, S. W., and Lin, A. W. (2000) Carcinogenesis 21, 485–495
3. Makin, G., and Dwek, C. (2001) Trends Cell Biol. 11, 822–826
4. O’Gorman, D. M., and Cotter, T. G. (2001) Leukemia (Baltimore) 15, 21–34
5. Toole, B. P. (2001) Semin. Cell Dev. Biol. 12, 79–87
6. Turley, E. A., Noble, P. W., and Bourguignon, L. Y. (2002) J. Biol. Chem. 277, 4589–4592
7. Ghatak, S., Misra, S., and Toole, B. P. (2002) J. Biol. Chem. 277, 38013–38020
8. Underhill, C. B., Chi-Rosso, G., and Toole, B. P. (1983) J. Biol. Chem. 258, 8086–8091
9. Lesley, J., Hasclall, V. C., Tammi, M., and Hyman, R. (2000) J. Biol. Chem. 275, 26967–26975
10. Li, R., Huang, L., Guo, H., and Toole, B. P. (2001) J. Cell Physiol. 186, 371–379
11. Ward, J., Huang, L., Guo, H., Ghatak, S., and Toole, B. P. (2003) Am. J. Pathol. 162, 1403–1409
12. Kengtawelert, P., and Ghosh, P. (1990) Anal. Biochem. 185, 313–318
13. Fairchild, C. R., Ivy, S. P., Kao-Shan, C. S., Whang-Peng, J., Rosen, N., Israel, M. A., Melera, P. W., Cowan, K. H., and Goldsmith, M. E. (1987) Cancer Res. 47, 5141–5148
14. Worm, J., Kirkin, A. F., Dhandhughayan, K. N., and Goldberg, P. (2001) J. Biol. Chem. 276, 39990–40000
15. Weigel, P. H., Hasclall, V. C., and Tammi, M. (1997) J. Biol. Chem. 272, 13997–14000
16. Eriwas, C., Zhang, Y., DeCastro, R., Guo, H., Nakamura, T., Kataoka, H., and Nageshima, K. (1995) Cancer Res. 55, 434–439
17. Zucker, S., Hynowitz, M., Rollo, E. E., Mann, R., Conner, C. E., Cao, J., Foda, H. D., Tompkins, D. C., and Toole, B. P. (2001) Am. J. Pathol. 158, 1921–1928
18. Bonni, A., Brunet, A., West, A. E. A., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Science 286, 1358–1362
19. Mabuchi, S., Ohmichi, M., Murata, A., Hisamoto, K., Hayakawa, J., Nishio, Y., Adachi, K., Takahashi, K., Arimoto-Ishida, E., Nakatsuji, Y., Tatsaka, K., and Murata, Y. (2002) J. Biol. Chem. 277, 33490–33500
20. Masters, S. C., Yang, H., Datta, S. R., Greenberg, M. E., and Fu, H. (2001) Mol. Pharmacol. 60, 1325–1331
21. Tamura, M., Gu, J., Tran, H., and Yamada, R. M. (1999) J. Natl. Cancer Inst. 91, 1820–1828
22. Almeda, E. A., Ilie, D., Han, Q., Hauck, C. R., Jin, F., Kawakatsu, H., Schlaepfer, D. D., and Damsky, C. H. (2000) J. Cell Biol. 149, 741–754
23. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2905–2927
24. Baugnart, G., Gomar-Hoss, C., Sakr, L., Ulesperger, E., and Wegrzinski, C. (1998) Cancer Lett. 131, 85–99
25. St. Croix, B., Man, S., and Kerbel, R. S. (1998) Cancer Lett. 131, 35–44
26. Desouze, E., and Jardillier, J. (2000) Crit. Rev. Oncol. Hematol. 36, 193–207
27. Underhill, C. B., and Toole, B. P. (1991) Exp. Cell Res. 131, 419–423
28. Underhill, C. B., and Toole, B. P. (1981) J. Biol. Chem. 256, 8086–8091
29. Huang, L., Guo, H., and Toole, B. P. (2001) J. Biol. Chem. 276, 39990–40000
30. Weigel, P. H., Hasclall, V. C., and Tammi, M. (1997) J. Biol. Chem. 272, 13997–14000
31. Eriwas, C., Zhang, Y., DeCastro, R., Guo, H., Nakamura, T., Kataoka, H., and Nageshima, K. (1995) Cancer Res. 55, 434–439
32. Zucker, S., Hynowitz, M., Rollo, E. E., Mann, R., Conner, C. E., Cao, J., Foda, H. D., Tompkins, D. C., and Toole, B. P. (2001) Am. J. Pathol. 158, 1921–1928
33. Bonni, A., Brunet, A., West, A. E. A., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Science 286, 1358–1362
34. Mabuchi, S., Ohmichi, M., Murata, A., Hisamoto, K., Hayakawa, J., Nishio, Y., Adachi, K., Takahashi, K., Arimoto-Ishida, E., Nakatsuji, Y., Tatsaka, K., and Murata, Y. (2002) J. Biol. Chem. 277, 33490–33500
35. Masters, S. C., Yang, H., Datta, S. R., Greenberg, M. E., and Fu, H. (2001) Mol. Pharmacol. 60, 1325–1331
36. Tamura, M., Gu, J., Tran, H., and Yamada, R. M. (1999) J. Natl. Cancer Inst. 91, 1820–1828
37. Almeda, E. A., Ilie, D., Han, Q., Hauck, C. R., Jin, F., Kawakatsu, H., Schlaepfer, D. D., and Damsky, C. H. (2000) J. Cell Biol. 149, 741–754
38. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2905–2927
39. Baugnart, G., Gomar-Hoss, C., Sakr, L., Ulesperger, E., and Wegrzinski, C. (1998) Cancer Lett. 131, 85–99
40. St. Croix, B., Man, S., and Kerbel, R. S. (1998) Cancer Lett. 131, 35–44
41. Desouze, E., and Jardillier, J. (2000) Crit. Rev. Oncol. Hematol. 36, 193–207
42. Underhill, C. B., and Toole, B. P. (1991) Exp. Cell Res. 131, 419–423
43. Damiano, J. S., Hazlehurst, L. A., and Dalton, W. S. (2001) Leukemia 15, 1222–1229
44. Frisch, S. M., and Scroenat, R. A. (2001) Curr. Opin. Cell Biol. 13, 555–562
45. Toole, B. P. (2002) Glycobiology 12, 37R–42R
46. Toole, B. P., and Hasclall, V. C. (2002) Am. J. Pathol. 161, 745–747
