Modulation of Anthracycline-Induced Myofibrillar Disarray in Rat Ventricular Myocytes by Neuregulin-1β and Anti-erbB2

Potential Mechanism for Trastuzumab-Induced Cardiotoxicity

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Background—There is an increased incidence of heart failure in patients treated concurrently with anthracyclines and the chemotherapeutic anti-erbB2 agent trastuzumab (Herceptin). On the basis of our previous studies with recombinant neuregulin-1β (NRG-1β), a ligand for the erbB2 receptor tyrosine kinase, we hypothesized that activation of erbB2 by anti-erbB2 versus NRG-1 would cause differential effects on myocyte intracellular signaling as well as anthracycline-induced myofibrillar injury and might potentially account for the clinical toxicity of trastuzumab in the setting of concurrent anthracycline therapy.

Methods and Results—We tested this hypothesis using adult rat ventricular myocytes (ARVMs) in culture, assessing myofibrillar structure by immunostaining for myomesin and filamentous actin. Activation of erbB2, extracellular signal–regulated kinase 1/2 (Erk1/2), and Akt was assessed by use of antibodies to phosphorylated activated receptor or kinase detected by immunoblot. ARVMs treated with doxorubicin (0.1 to 0.5 μmol/L) showed a concentration-dependent increase in myofilament disarray. NRG-1β (10 ng/mL) activated erbB2, Erk1/2, and Akt in ARVMs and significantly reduced anthracycline-induced disarray. In contrast to NRG-1β, anti-erbB2 (1 μg/mL) caused rapid phosphorylation of erbB2 but not Erk1/2 or Akt, with downregulation of erbB2 by 24 hours. Concomitant treatment of myocytes with anti-erbB2 and doxorubicin caused a significant increase in myofibrillar disarray versus doxorubicin alone.

Conclusions—NRG-1β/erbB signaling regulates anthracycline-induced myofilament injury. The increased susceptibility of myofilaments to doxorubicin in the presence of antibody to erbB2 may explain the contractile dysfunction seen in patients receiving concurrent trastuzumab and anthracyclines. (Circulation. 2002;105:1551-1554.)

Key Words: erbB2 ▶ cardiotoxicity ▶ neuregulins ▶ anthracyclines ▶ myocytes

Treatment of metastatic breast cancer with anthracyclines and trastuzumab, a novel therapy derived from an antibody to the erbB2 receptor tyrosine kinase, results in a marked increase of left ventricular dysfunction and symptomatic heart failure.1 ErbB2 is a member of the epidermal growth factor receptor family, and along with neuregulin and the erbB4 receptor, it plays an essential role in cardiac development.2–4 We have shown that recombinant neuregulin 1β (NRG-1β) activates both erbB2 and erbB4 receptor tyrosine kinase activity and promotes growth, myofilament organization, and survival of isolated cardiac myocytes.5,6 The clinical observation of the cardiotoxicity of trastuzumab and anthracyclines suggests that the neuregulin/erbB system modulates the response of the myocardium to anthracyclines. Possible mechanisms for this toxicity are alterations in the structure,7 gene expression,8 and survival9 of cardiac myocytes. The main purpose of this study was to test the hypothesis that trastuzumab alters the susceptibility of myocytes to anthracycline-induced myofibrillar disarray. We therefore characterized the effect of anthracyclines on myocyte myofibrillar structure in isolated adult rat cardiac myocytes in primary culture and examined the effect of NRG-1β and an antibody to erbB2 with properties similar to trastuzumab10 on erbB2 signaling and anthracycline-induced changes in myofibrillar structure.

Methods

Chemicals
Recombinant NRG-1α and NRG-1β were purchased from NeoMarkers, as were biological-grade antibodies to rodent erbB2 (Clone...
Preparation of Cardiac Myocytes

Adult rat ventricular myocytes (ARVMs) were isolated from female Sprague-Dawley rats weighing 150 to 200 g as previously described. Culture medium was based on M-199 (Amiremed or Gibco-BRL) and contained 20 mmol/L creatine (Sigma), 1% 100-U/mL penicillin/streptomycin (Gibco-BRL), 10% preselected FCS (Seramed), and 10 μmol/L cytosine arabinoside (Sigma-Aldrich). ARVMs were treated on day 7 in culture.

Immunofluorescence Microscopy

Cell cultures were fixed and myomesin and filamentous actin were stained as previously described with primary antibody to myomesin, secondary antibodies conjugated to FITC or cyanine-5 from Jackson Immunoresearch, and rhodamine-phalloidin from Molecular Probes. Stained preparations were analyzed with a Leica confocal scanner TCS NT on the inverted microscope Leica DMIRB-E. Myofibrillar disarray was assessed by an investigator blinded to treatment using a Zeiss Axioplan fluorescence microscope equipped with a 63 times oil immersion objective. A total of 200 to 250 myocytes were counted for each experimental condition in each experiment.

Detection of Erk1/2, Akt/PKB, erbB2, and erbB4 Phosphorylation

Activated Erk1/2 and Akt were detected with a PhosphoPlus Akt/PKB (Ser473) antibody kit and p44/42 MAP kinase assay kit (New England BioLabs Inc) as previously described. Separate membranes were probed with an anti-Akt/PKB and anti-erb1/2 antibodies to ensure equal loading. Activation of the erbB2 receptor tyrosine kinase was detected as previously described by immunoprecipitation from volumes of cell lysates in RIPA buffer (500 μg total protein) with antibodies to erbB2 and immunodetection using anti-phosphotyrosine antibody (Santa Cruz Biotechnology), horseradish peroxidase–conjugated goat anti-mouse secondary antibody (Sigma-Aldrich), and chemiluminescence detection.

Results

The effect of doxorubicin on myofilament structure was examined in redifferentiated ARVMs at day 7 in culture. Cells were treated with 0.1 or 0.5 μmol/L doxorubicin for 48 hours. Confocal microscopy of doxorubicin-treated myocytes showed the appearance of “moth-eaten,” “lattice-like” myofilament structure, with a relative paucity of myomesin staining (Figure 1, a through d). This occurred in the presence of intact chromatin structure, because simultaneous TUNEL staining of myocytes showed no evidence of DNA damage in these myocytes (data not shown). Doxorubicin treatment resulted in a concentration-dependent increase in the number of myocytes with evidence of disarray (Figure 1e). Cells viewed by time-lapse microscopy and subsequent immunofluorescence after fixation showed that myocytes with evidence of myofilibrillary disarray continued to beat at the time of fixation (data not shown).

To test whether erbB2 modulates anthracycline-induced cardiotoxicity, we examined the effect of NRG-1β and a monoclonal antibody to rat erbB2 on anthracycline-induced myofibrillar disarray. Like NRG-1β, treatment of ARVMs with anti-erbB2 (1 μg/mL) for 10 minutes induced tyrosine phosphorylation of myocyte erbB2, consistent with receptor activation (Figure 2a). After 24 hours of exposure to the antibody, readdition of antibody caused no further activation of erbB2, consistent with downregulation of erbB2 by this antibody. Activation of erbB2 by anti-erbB2 had no effect on activation of Erk1/2 or Akt, in contrast to NRG-1β, which activates both kinases (Figure 2b). Treatment of ARVMs simultaneously with NRG-1β (50 ng/mL) and doxorubicin decreased the number of myocytes showing evidence of disarray (Figure 2c). There was no significant effect of NRG-1α on baseline or doxorubicin-induced disarray (data not shown). In contrast, treatment with anti-erbB2 and doxorubicin increased the number of myocytes showing a pattern of disarray (Figure 2d). Neither NRG-1β nor anti-erbB2 alone had any effect on myofilibrillary disarray under control conditions (data not shown).
Discussion

The erbB2 receptor tyrosine kinase is expressed in the myocardium by ventricular myocytes and is essential for normal cardiac development. Activation of erbB2 and erbB4 by recombinant NRG-1/H9252 leads to myocyte growth and inhibition of apoptosis in vitro. The present findings support a role for the NRG/erbB system in modulating myocardial response to anthracycline-induced injury. Moreover, these observations suggest a potential mechanism for the clinical observation of increased cardiotoxicity of anthracyclines in patients receiving trastuzumab, a monoclonal antibody to erbB2.

Anthracycline-induced myocardial injury is accompanied by an increase in myofibrillar disarray that is seen in patients, animals treated with anthracyclines in vivo, and in vitro studies of cardiac myocytes. The close relationship between myocyte ultrastructural damage and contractile dysfunction after anthracycline therapy suggests a mechanistic relationship. We found that even myocytes with evidence of myofibrillar degradation continue to beat in culture, although presumably these damaged cells generate less force. That these cells are viable, without evidence of DNA fragmentation, suggests that this damage may be reversible. It is also interesting that myofibrillar damage after anthracycline treatment both in vivo and in vitro (our own data, as well as Reference 7) is patchy, with affected myocytes seen adjacent to structurally normal myocytes. This suggests that the effect of anthracyclines on myofibrillar structure is at least in part controlled locally.

The NRG/erbB system may be one form of local control. NRG-1β is expressed in microvascular endothelial cells and acts on the erbB2 and erbB4 receptors to alter myofilament structure in isolated myocytes via a phosphatidyl inositol 3 kinase–dependent pathway. Although both anti-erbB2 and NRG-1/H9252 induced similar degrees of phosphorylation of the erbB2 receptor in the short term, they had opposite effects on doxorubicin-induced changes in myofilament structure. The deleterious effect of anti-erbB2, conversely, might occur through downregulation of erbB2 expression, suppression of intracellular signaling, or other mechanisms.

It is important to note that most women receiving trastuzumab without concurrent anthracyclines did not develop left ventricular dysfunction or overt heart failure. This suggests heterogeneity in the baseline activity of the NRG/erbB system among individuals, resulting from either genetic predisposition or, more likely, environmental stress. Our own data in vitro with anti-erbB2 support the latter conclusion, because we saw a deleterious effect of anti-erbB2 only in the presence of anthracyclines, arguably a source of “stress.” The variability in the clinical toxicity of trastuzumab may thus be a result of various degrees of superimposed hemodynamic or other stress during trastuzumab therapy. Carefully monitored use of trastuzumab in cancer victims with other cardiovascular...
lar conditions, including aortic stenosis, hypertension, and ischemic heart disease, will help to address this hypothesis.

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