Fluid shear stress remolds expression and function of junctional proteins in cultured bone cells

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Submitted 1 February 2002; accepted in final form 30 September 2002

Thi, Mia M., Takashi Kojima, Stephen C. Cowin, Sheldon Weinbaum, and David C. Spray. Fluid shear stress remodels expression and function of junctional proteins in cultured bone cells. Am J Physiol Cell Physiol 284: C389–C403, 2003. First published October 3, 2002; 10.1152/ajpcell.00052.2002.—We tested the hypothesis that fluid shear stress (τ) modifies the expression, function, and distribution of junctional proteins [connexin (Cx)43, Cx45, and zona occludens (ZO)-1] in cultured bone cells. Cell lines with osteoblastic (MC3T3-E1 cells) and osteocytic (MLO-Y4 cells) phenotypes were exposed to τ-values of 5 or 20 dyn/cm2 for 1–3 h. Immunostaining indicated that at 5 dyn/cm2, the expression and function of Cx43 and Cx45, and zona occludens (ZO-1) was selectively up- and downregulated in response to different shear stress levels. These results indicate that in cultured bone cells, fluid shear stress disrupts junctional communication, rearranges junctional proteins, and determines de novo synthesis of specific connexins to an extent that depends on the magnitude of the shear stress. Such disconnection from the bone cell network may provide part of the signal whereby the disconnected cells or the remaining network initiate focal bone remodeling.

Previous experimental studies have shown that bone cells release signaling molecules such as prostaglandins, nitric oxide (NO), Ca2+, and other second messengers in response to the fluid shear stress (3, 18, 20, 24, 41).

One mechanism of bone remodeling by which second messenger signals spread throughout the bone cell network involves gap junction channels that connect osteoblasts and bone-lining cells along the surfaces of Haversian and Volkmann canals and osteocytes that are embedded within the bone matrix (15, 22, 62). The connexin proteins that form gap junctions are encoded by a gene family with as many as 21 members in mammals (56). Connexins are expressed with an overlapping pattern of tissue specificity; connexin43 (Cx43) and connexin45 (Cx45) are the gap junction proteins that have been associated with bone cells (5, 33, 34, 48, 57).

Gap junction channels, including those formed by Cx43 and Cx45, are permeable to signaling ions and second messenger molecules (46). Gap junctional communication of such signals between bone cells gives rise to modulation of hormonal responses in the osteoblastic network (53), regulation of gene expression (29), and propagation of intracellular signals (12). Moreover, connexin expression and function are essential for normal osteogenesis and bone mineralization (30, 31, 34).

Connexins may also play roles in addition to the formation of pathways for intercellular communication. Recent studies have shown that connexins directly interact with both adherens and tight junction-associated proteins including zona occludens-1 (ZO-1), claudins, and β-catenin (1, 16, 26, 37, 50). Accordingly, it has been proposed that connexins are part of a multimolecular signaling complex, the “Nexus” (47). From the standpoint of the studies described here, ZO-1 has been shown to interact with gap junction proteins Cx43 and Cx45 in osteoblastic cells (28) where ZO-1 binding to connexins may play a role in the...
organization, trafficking, and/or stabilization of gap junction proteins (28, 49).

Regulation of gap junctional communication in response to load-induced biophysical signals has been examined in both vascular endothelial (6, 10) and bone cells (4, 13, 62, 63). Although such studies have generally reported an increase in Cx43 expression, changes in functional coupling in the bone cell network have not been as consistently demonstrated.

In this study, we tested the hypothesis that fluid shear stress of the magnitude that is expected to occur in bone tissue modifies the expression, distribution, and function of connexins (Cx43 and Cx45) and an associated protein (ZO-1). Well-characterized cell lines, MC3T3-E1 osteoblastic and MLO-Y4 osteocytic cells, were used in our experiments. Our results demonstrate that in both osteoblastic and osteocytic cell lines, fluid shear stress disrupts cell-to-cell junctional communication, rearranges gap junction proteins, and determines de novo synthesis of specific connexins to an extent that depends on the magnitude of the shear stress. Such disconnection from the bone cell network due to fluid shear stress may provide part of the signal whereby the disconnected cells or the remaining network initiates focal bone remodeling.

**MATERIALS AND METHODS**

**Cell culture.** Osteoblastlike MC3T3-E1 cells (obtained from Dr. Kenneth J. McLeod, SUNY, Stony Brook) were cultured in α-MEM (GIBCO-BRL, Grand Island, NY) that contained 1% penicillin-streptomycin (GIBCO-BRL) and 5% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA), and osteocyte-like MLO-Y4 cells (obtained from Dr. Lynda F. Bonewald, Univ. TX Health Science Center) were cultured in osteocyte-like MLO-Y4 cells (obtained from Dr. Lynda F. Bonewald, Univ. TX Health Science Center) were cultured in α-MEM that contained 1% penicillin-streptomycin, 10% FBS, and 2.5% calf serum (GIBCO-BRL) at 37°C with 95% O2-5% CO2. For each cell type, confluent monolayers of cells were grown on glass slides in static conditions and transferred to the flow apparatus to expose the monolayers to a fluid τ-value of 5 or 20 dyn/cm2 for 1, 2, or 3 h. These durations were chosen based on the turnover rates of Cx43 and Cx45, which exhibit half-lives of 1.5 and 3 h, respectively (9).

**Flow chamber and experiment.** The fluid-flow setup consisted of a parallel-plate flow chamber (Cytodyne, La Jolla, CA) and a recirculating flow circuit. This circuit included a variable-speed peristaltic pump (Taitec, Saitama, Japan), pulse dampener (Cole-Palmer Instruments, Vernon Hills, IL), and a reservoir with culture medium (α-MEM with 1% FBS) maintained at 37°C with 95% O2-5% CO2. This system produces laminar flow over a cell monolayer. A flow rate was chosen to yield a τ-value of 5 or 20 dyn/cm2 using the equation $\tau = 6\mu Q h^2$, where Q is flow rate, $\mu$ is medium viscosity, and b and h are channel width and height, respectively. Control cells were kept under static conditions with the same culture medium at 37°C.

**Alkaline phosphatase staining.** To determine whether cells retained the differentiated phenotypes, both cell lines were routinely checked for alkaline phosphatase activity. Cells were fixed with 4% formaldehyde and permeabilized with 70% ethyl alcohol (EtOH). Cells were then rinsed with 0.2 M Tris(hydroxymethyl)-aminomethane (Sigma, St. Louis, MO) and incubated in naphthol AS-BI phosphate (Sigma) and fast red violet LB salt (Sigma) plus Tris-HCl for 30 min (2). After several washes with distilled water, cells were counterstained with Mayer’s hematoxylin (Sigma) for 2 min. The cells were then washed with distilled water and mounted for analysis.

**Cell viability studies.** Cells from both control and shear-stress-exposed samples were analyzed for health and viability using the Live/Dead Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR). Cells were rinsed a few times with 1× PBS and incubated with 4 μM ethidium homodimer-1 (EtD-1) and 2 μM of calcine-acetoxymethyl ester in 1× PBS for 30 min as recommended by the manufacturer. Live cells, which retained the polyanionic dye calcine, gave rise to green fluorescence; dead cells, which took up EtD-1 through membrane damage, produced red fluorescence. Live and dead cells were counted from 10 cell fields for all of the samples, and percentages of live and dead cells were calculated.

**Immunofluorescence studies.** Both control and shear-stress-exposed cells were fixed with 2% formaldehyde, permeabilized with 0.4% Triton X-100 (Sigma), and blocked with 10% goat serum (GIBCO-BRL) in 1× PBS. The cells were then incubated with primary polyclonal antibodies against Cx43, Cx45 (courtesy of Dr. E. Hertzberg, AECOM and Dr. T. Shaw, Univ. Washington, School of Medicine, Seattle, WA), and secondary antibody conjugated to Alexa 488 (Molecular Probes). For F-actin staining, cells were incubated with rhodamine-labeled phalloidin (Sigma) immediately after fixation. The coverslips were mounted on slides, examined on a Nikon Eclipse TE300 microscope, and photographed using a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

**Western blot analysis.** Controls and shear-stress-exposed (1 and 3 h) samples were lysed in 80 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM phenylmethylsulfonyl fluoride (PMFSF), sonicated, and centrifuged (14,000 rpm for 30 min) as described by Guan et al. (17). Pellets and supernatants from the samples were collected for crude membrane and cytosolic protein analyses. Samples were loaded onto 10% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) for separation and were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). The membranes were probed with primary polyclonal and monoclonal antibodies to Cx43, Cx45, and ZO-1, polyclonal β-actin (Sigma), and monoclonal GAPDH (Research Diagnostics, Flanders, NJ), followed by secondary antibody incubation with horseradish peroxidase (HP)-conjugated anti-rabbit and anti-mouse IgGs (Santa Cruz Biotech, Santa Cruz, CA). The protein bands were detected using the Amersham ECL detection kit (Amersham Biosciences, Piscataway, NJ) and were exposed on Fuji X-ray film. The intensity of the bands was analyzed using Scion NIH Image software (Scion, Frederick, MD). Measured intensities for all experiments were first normalized with respect to internal controls (GAPDH for cytosolic proteins and β-actin for membrane-bound proteins) and then with respect to controls.

**Northern blot analysis.** Total RNA from the samples was extracted using TRIzol reagent (GIBCO-BRL) and was quantified as previously described (52). Total RNA (10 μg) from the samples was separated on 1.2% formaldehyde-agarose gel and was transferred onto a Gene Screen hybridization transfer membrane (NEN Life Science Products, Boston, MA). Membranes were hybridized with appropriate denatured random-primer probes. The rat cDNA probes used were full-length Cx43 and Cx45 (obtained from Dr. Eric Beyer, Univ. of Chicago Medical School) and 18S labeled with [32P]dCTP using the Megaprime labeling system (Amersham Biosciences). The membranes were then exposed to the phosphor screen overnight, scanned on a Storm PhosphorImager system, and quantified using ImageQuant software (Molecu-
lar Dynamics, Sunnyvale, CA). All acquired data were first normalized with respect to 18S RNA band intensity, and then all experimental data were normalized with respect to control data.

RT-PCR and semiquantitative RT-PCR analyses. RT-PCR was performed as previously described (52). For semiquantitative PCR, a 9:1 ratio of competimers and 18S primers (Ambion, Austin, TX) was added to the PCR mixture. Conditions applied for PCR using a PTC-100 programmable thermal controller (MJ Research, Watertown, MA) were 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 30 cycles, and 72°C for 8 min. Reaction products were analyzed by electrophoresis on 2% agarose gels and were quantified using Kodak 1D Scientific Imaging Systems. The following primers were used for each cDNA amplification: mCx43, TACCACGCCACCACC TGGC (sense), AATCTCCAGGTCATC AGG (antisense); mCx45, AAAGAGGAGGCAACCAA (sense), GTCCCAAACCCTAAGTG AAGC (antisense) (11); mZO-1, CATAGAATAGACTCCCCTTG (sense), GCTTGAGACGTCCCAAACCCTAAGTG AAGC (antisense) (21); and mosteocalcin, gcagacgctcattgcacag (sense), TTTGAGACCCTGAGACGC CGAAA (antisense) (23).

Scrape loading. Quantitative scrape loading as described by Pina-Benabou et al. (40) was used to analyze gap junctional communication after cells were exposed to shear stress. Incisions on the cell monolayers of control and shear stress-exposed samples were made with a fine razor blade in two or three different regions on the slides. The slides were incubated with 0.5% Lucifer yellow (Sigma) at 37°C, rinsed with 1× PBS, and fixed with 2% formaldehyde. The intensity of the dye spread from the damaged cells to the neighboring cells was observed using a Nikon Eclipse TE300 microscope and was photographed with a SPOT-RT digital camera. Extent of dye spread was quantified as linear distance perpendicular to the scrape using Scion NIH Image software.

Statistical analysis. Data were analyzed using one-way ANOVA (SigmaStat, Chicago, IL). Northern blot analyses are presented as means ± SE of six experiments. Western blot, quantitative RT-PCR, and scrape-loading analyses are presented as means ± SE of three experiments. A significant difference compared with controls is indicated as * P < 0.05.

RESULTS

Osteoblast and osteocyte cell lines express gap junction proteins associated with osteogenesis and tight junction-associated protein ZO-1. We used the osteoblastic cell line MC3T3-E1 (57) and the osteocyte cell line MLO-Y4 (23) to compare expression, distribution, and function of gap junctions associated with osteogenesis (34). As shown in Fig. 1A in which the cell cytoskeleton was stained with phalloidin, these cell lines show quite different morphologies. Whereas the osteoblast cells exhibit flattened, epitheloid shapes and a close packing arrangement, the osteocyte cells exhibit a more stellate shape with rounded somata and multiple processes extending for variable distances to neighboring cells. Because phenotypic expression of differentiated cell markers may vary in cell lines maintained under different conditions and after prolonged passage, we determined expression levels of alkaline phosphatase and osteocalcin in the MC3T3-E1 and MLO-Y4 cell lines under conditions used in our studies. Alkaline phosphatase activity was higher in the osteoblasts than in the osteocytes (Fig. 1B), whereas the osteocytic cell line but not the osteoblastic cell line expressed measurable osteocalcin mRNA (Fig. 1C). RT-PCR results showed that under normal conditions, both types of immortalized cell lines expressed mRNAs corresponding to osteogenic gap junction proteins connexin (Cx)43 and Cx45 and the tight junction-associated protein zona occludens (ZO)-1.

Fluid shear stress does not affect cell viability but disrupts cell-to-cell communication and rearranges gap junction proteins Cx43 and Cx45 and associated ZO-1 protein in cultured bone cells. The overall experimental design critically depends on maintenance of cell viability during periods of shear stress exposure. To determine the extent to which cells were injured by the procedure, we performed the Live/Dead assay on both cell types with no shear stress and with 5 and 20
dyn/cm² of shear stress for 3 h. Both MC3T3-E1 and MLO-Y4 cells were viable after 3 h of exposure to fluid shear stress. As shown, applying this assay to osteoblastic (Fig. 2A) and osteocytic (Fig. 2B) cells revealed that the percentages of live cells in normal and shear stress-exposed cells were all >80%. ANOVA analysis revealed that the percentage of dead cells was lower than live cells for controls and for each treatment, but the percentage of dead cells did not significantly differ among the groups.

Immunofluorescence microscopy was used to analyze the effect of fluid shear stress on the distribution and arrangement of Cx43, Cx45, and ZO-1 at cell borders as well as in perinuclear areas in cultured osteoblast and osteocyte cell lines. Under control (no-flow) conditions, MC3T3-E1 cells possessed extensive contact with neighboring cells. Immunostaining of control MC3T3-E1 cells revealed abundant punctate and linear appositional staining of Cx43 and ZO-1 at cell borders as well as Cx43 immunoreactivity in perinuclear regions; both...
Cx43 and ZO-1 also showed diffuse cytoplasmic staining throughout the osteoblastic cells (Fig. 3A). Overlays of images acquired from cells double-labeled for Cx43 and ZO-1 (Fig. 4A) indicated that overlap was virtually complete at membrane appositions, although more Cx43 was detected intracellularly. Wispy Cx45 immunostaining was also present at appositional regions, although it was mainly concentrated at perinuclear regions within the cells (see Fig. 3A).

Exposure of MC3T3-E1 osteoblast cells to both levels of shear stress resulted in changes in cell morphology (see Fig. 3, B and C) and in redistribution of connexins and ZO-1 within the cells. Even as early as 1 h after exposure to 5 dyn/cm² of shear stress, cells began to
elongate with respect to flow direction and lost the majority of direct cell-body contact with neighboring cells; such anisotropy was even more apparent after longer duration exposures to higher shear stress. Shear stress rapidly affected Cx43 distribution in osteoblastic cells when MC3T3-E1 cells were exposed to a \( \tau \)-value of 5 dyn/cm\(^2\). The distribution of Cx43 was moderately disrupted at cell membranes with increased perinuclear and cytoplasmic staining being detectable as early as within 1 h of exposure time (data not shown). Increased perinuclear and cytoplasmic staining for Cx43 with concomitant additional reduc-

Fig. 4. Fluid shear stress dramatically reduced the colocalization of Cx43 and ZO-1 in MC3T3-E1 and MLO-Y4 cells. Cells were subjected to low (\( \tau = 5 \) dyn/cm\(^2\)) and high (\( \tau = 20 \) dyn/cm\(^2\)) shear stress levels for 1 or 3 h, and control cells were kept in static conditions. Effects of fluid shear stress on the colocalization of Cx43 and ZO-1 in MC3T3-E1 (A) and MLO-Y4 (B) cells were analyzed using immunofluorescence microscopy. Formaldehyde-fixed, Triton X-100-permeabilized samples were double-labeled with primary polyclonal and monoclonal antibodies against Cx43 and ZO-1 followed by fluorescein-conjugated goat anti-rabbit and anti-mouse IgGs. Flow direction (arrows) and colocalization of Cx43 and ZO-1 (arrowheads) are indicated, bar, 20 \( \mu \)m.
tion in appositional staining was even more apparent at 3 h of exposure at 5 dyn/cm² of shear stress (see Fig. 3B). Although ZO-1 distribution was also altered by flow, it was altered differently than Cx43. At 1 h of low shear stress, ZO-1 distribution was largely appositional (data not shown), which was similar to the controls, whereas disruption was observed only at 3 h (see Fig. 3B). In contrast to Cx43, diffuse cytoplasmic ZO-1 staining was only occasionally detectable. Exposure to 20 dyn/cm² of shear stress for both 1 and 3 h produced more pronounced disruption of both Cx43 and ZO-1 at the cell membrane and a moderate increase of Cx43 perinuclear and cytoplasmic staining (see Fig. 3C). Overlay images of Cx43 and ZO-1 from shear stress-exposed samples clearly show a dramatic reduction in overlap of these proteins at the contact cell borders (Fig. 4A). With regard to Cx45, low shear stress (especially at 3 h) led to reduced appositional and increased cytoplasmic staining; this change in Cx45 distribution was more prominent at 20 dyn/cm² of shear stress (see Fig. 3C).

Control MLO-Y4 osteocyte cells possessed rounded cell bodies with numerous cell processes and were connected to neighboring cells via these processes. Immunostaining of these control cells revealed that Cx43 and ZO-1 were concentrated at the tips of the cell processes at regions of contact with neighboring cells, with Cx43 also showing perinuclear distribution (see Fig. 3D). Similar to MC3T3-E1 cells, colocalization of Cx43 and ZO-1 in control MLO-Y4 cells indicated that overlap was found at the tips of opposing cell processes (Fig. 4B). For Cx45, there was very faint staining at the tips of some opposing processes of the control cells (see Fig. 3D). Dramatic morphological changes were observed at both low and high levels of shear stress. The cell processes became smaller in diameter and somewhat more numerous, whereas the cell bodies became smaller and more rounded. When MLO-Y4 cells were subjected to 5 dyn/cm² of shear stress for 1 or 3 h, Cx43 and ZO-1 at the tips of opposing cell processes were moderately decreased, whereas perinuclear distribution of Cx43 notably increased (see Fig. 3E). At 20 dyn/cm² of shear stress, significant disruption of both Cx43 and ZO-1 at the opposing membrane tips was observed for both exposure durations (see Fig. 3F, which illustrates results for 3 h) with a slight increase in cytoplasmic and perinuclear staining of Cx43. Colocalization of Cx43 and ZO-1 was also reduced at the opposing tips of the cell processes after exposure to τ-values of both 5 and 20 dyn/cm² (Fig. 4B). Immunofluorescence for Cx45 indicated that perinuclear distribution became more prominent as shear stress increased (see Fig. 3, E and F). These results suggest that both levels of fluid shear stress reorganize the distribution of junctional proteins as early as within 1 h of exposure time, which would be expected to produce an effect on junctional communication.

**Intercellular coupling in cultured bone cells is significantly decreased by fluid shear stress.** Previous studies (4, 57) have shown that MC3T3-E1 and MLO-Y4 cells are coupled by functional gap junction channels. In this study, we used the scrape-loading technique to quantitatively examine the effects of fluid shear stress on cell-to-cell coupling. Our scrape-loading data indicated that intercellular coupling significantly decreased in MC3T3-E1 cells; this change was more pronounced with higher shear stress and longer duration. As shown in Fig. 5A, the extent of dye spread in control MC3T3-E1 cells was 270.4 ± 10.7 μm. This dye-transfer distance was reduced to 237.4 ± 4.7 or 120.8 ± 5.0 μm when cells were exposed to the lower τ-value of 5 dyn/cm² for 1 or 3 h, respectively. At the high τ-value of 20 dyn/cm², the degree of dye transfer was dramatically reduced to 168.4 ± 4.7 μm for the shorter duration and 106 ± 6.3 μm for the longer duration (Fig. 5, bar graphs).

MLO-Y4 cells showed more uniform changes in dye coupling when exposed to shear stress. As shown in Fig. 5B, the average dye-transfer distance for control MLO-Y4 cells was 109.5 ± 4.3 μm. When cells were exposed to 5 dyn/cm² of shear stress for 1 or 3 h, this dye-transfer distance was significantly decreased to 72 ± 4.9 or 62.1 ± 3.7 μm (Fig. 5, bar graphs). Similar decreases were observed with the samples that were exposed to a high τ-value of 20 dyn/cm² for 1 or 3 h. These data imply that both levels of fluid shear stress inhibited intercellular coupling, which in turn supports the conclusion that both levels also disrupt cell-to-cell junctional communication and redistribute the junctional proteins depending on the duration and level of shear stress.

**Fluid shear stress downregulates ZO-1 and phosphorylated Cx43 in cultured bone cells.** We performed Western blot analyses for crude membrane and cytosolic proteins to determine the extent to which fluid shear stress regulates levels of Cx43, Cx45, and ZO-1 within each cell type. For Cx43, multiple bands were detected in Western blots corresponding to different extents and/or types of phosphorylation (35, 36), where NP is the dephosphorylated form and P1 and P2 designate phosphorylated Cx43 species. For both cell lines at all shear stress levels, there was a similar pattern in which the P2 form of Cx43 from the membrane was significantly decreased in the membrane fraction, whereas all three forms of Cx43 were dramatically increased in the cytosolic fraction. For osteoblastic MC3T3-E1 cells, densitometric analysis of membrane-bound Cx43 bands showed significant downregulation of the P2 form of Cx43 at 3 h for both 5 and 20 dyn/cm² of shear stress. By contrast, NP, P1, and P2 forms of cytosolic Cx43 increased after exposure to both levels of shear stress (Fig. 6A). Similarly, densitometric analysis of membrane-bound Cx43 for MLO-Y4 cells revealed downregulation of the P2 form at both 5 and 20 dyn/cm² of shear stress, whereas cytosolic Cx43 noticeably increased in response to both levels of shear stress, especially to 5 dyn/cm² (Fig. 6B). In both MC3T3-E1 and MLO-Y4 cells, Cx45 was detectable only in the cytosolic fraction, and low levels of shear stress seemed to downregulate Cx45, whereas high levels of shear stress appeared to upregulate Cx45 at 20 dyn/cm² of shear stress (Fig. 6B).
both exposure times (Fig. 7A). Western blots using ZO-1-specific antibodies revealed that for both shear stress levels, this membrane-bound protein was down-regulated at longer exposure times in both MC3T3-E1 and MLO-Y4 cells (Fig. 7B). These data indicate that fluid shear stress regulates the level of Cx45 and ZO-1 expression and abundance of all three forms of Cx43. Notably, the P2 form of the membrane-bound Cx43 decreased as the duration of the shear stress increased. Such a correlation of decreased P2 with decreased dye coupling is consistent with previous reports on other cell types (see DISCUSSION).

Selective regulation of Cx43, Cx45, and ZO-1 expression in response to different shear stress levels. Previous studies on endothelial cells have shown that fluid flow and mechanical stretch alter the expression of Cx43 (6, 10). Therefore, in this study, we analyzed the consequence of fluid shear stress on the expression of Cx43, Cx45, and ZO-1 mRNAs in osteoblastic and osteocytic cell lines using Northern blot analysis and semiquantitative RT-PCR. In both cell lines, different levels of shear stress seemed to regulate expression of Cx43 and Cx45 in a reciprocal or compensatory manner. As shown in Fig. 8A, Cx43 mRNA levels increased 1.5-2-fold for MC3T3-E1 cells and 1.25-1.5-fold for MLO-Y4 cells at 5 dyn/cm² of shear stress for 1, 2, or 3 h, yet no apparent changes were found from the controls at 20 dyn/cm² of shear stress for any of the durations for either cell types. By contrast, Cx45 mRNA remained the same as the controls at the lower shear stress level for all durations and increased to 1.25-1.75-fold at 20 dyn/cm² of shear stress for 1, 2, or 3 h (Fig. 8B). With regard to ZO-1 mRNA, there were no detectable changes with respect to control in MC3T3-E1 cells at the lower shear stress level, whereas the mRNA level was decreased to half that of controls at the higher shear stress level (Fig. 9A). However, more dramatic effects on ZO-1 expression were seen in MLO-Y4 cells, where ZO-1 mRNA was profoundly decreased at both 5 and 20 dyn/cm² of shear stress for 1-, 2-, or 3-h exposure times (Fig. 9B). These data suggest that for both cell lines, lower shear stress (5 dyn/cm²) upregulates the expression of Cx43, whereas higher...
shear stress (20 dyn/cm²) upregulates the expression of Cx45. By contrast, ZO-1 is downregulated at high shear stress levels in the osteoblastic cell line and even more strikingly at both shear stresses in the osteocytic cell line.

DISCUSSION

In this study, we found that steady fluid shear stress modifies expression, function, and distribution of connexins (Cx43 and Cx45) and an associated tight junc-
tion protein (ZO-1) in cultured bone cells. Our results strongly suggest that fluid shear stress disrupts cell-to-cell communication and rearranges the gap junction protein Cx43 and Cx45 and an associated protein ZO-1 in both MC3T3-E1 and MLO-Y4 cells. This disrupted gap junctional communication and rearrangement of Cx43, Cx45, and ZO-1 depended on the magnitude of the shear stress as well as the exposure duration. Our results as well as others (4, 14, 23, 57, 58) indicate that the major gap junction protein that mediates cell-to-cell communication in both cell types is Cx43. Our finding that the selective reduction in the

Fig. 7. High-magnitude shear stress upregulates cytosolic Cx45 whereas both levels of shear stress downregulate membrane-bound ZO-1 in cultured bone cells. Effects of fluid shear stress on the protein levels of Cx45 and ZO-1 were determined using Western blot analysis. Cells were lysed, and membrane-bound and cytosolic proteins were prepared from controls (lanes 1 and 4), 1-h exposure of $\tau = 5$ dyn/cm$^2$ (lane 2), 3-h exposure of $\tau = 5$ dyn/cm$^2$ (lane 3), 1-h exposure of $\tau = 20$ dyn/cm$^2$ (lane 5), or 3-h exposure of $\tau = 20$ dyn/cm$^2$ (lane 6) cells. Western blot analysis was performed using antibodies against Cx45, GAPDH (A), ZO-1, and $\beta$-actin (B) for MC3T3-E1 and MLO-Y4 cells. Densitometric analyses of cytosolic Cx45 and membrane-bound ZO-1 proteins from three independent experiments were performed using Scion NIH Image software. All acquired data were first normalized with respect to internal controls ($\beta$-actin for membrane-bound and GAPDH for cytosolic proteins) and then normalized with respect to control data. All data are presented as means $\pm$ SE, $n = 3$; *$P < 0.05$. 
P2 form of membrane-bound Cx43 correlates with the loss of dye coupling is consistent with previous reports, which indicate that Cx43 phosphorylation is important for its function (35, 36). Elevated levels of all three forms (NP, P1, and P2) of cytosolic Cx43 after exposure to fluid shear stress suggest that newly synthesized as well as internalized Cx43 contributed to the increases in cytosolic Cx43 level. We believe that this significant increase in cytosolic Cx43 level was mainly due to the internalization of membrane-bound Cx43, because newly synthesized Cx43 should mostly be in NP form.

Furthermore, based on the cytosolic Cx43 analysis, low shear stress seemed to be regulating Cx43 in both cell types, whereas high shear stress appeared to be up-regulating cytosolic Cx45. At the level of mRNA, Cx43, Cx45, and ZO-1 all showed interesting changes with fluid-induced shear stress. Expression of Cx43 and Cx45 mRNA was selectively upregulated in response to different shear stress levels, whereas both magnitudes of shear stress inhibited ZO-1 expression. Together,
in a manner that depends on the magnitude of the shear stress.

There is increasing evidence that fluid shear stress regulates Cx43 in vascular smooth muscle (6), vascular endothelial (10), and cultured bone cells (4). Our observation of the disruption and translocation or internalization of Cx43 from the membrane after exposure to laminar flow in both MC3T3-E1 and MLO-Y4 cells is in agreement with the findings of DePaola and coworkers (10) for endothelial cells in the laminar flow region at 5 h of exposure time. We speculate that fluid shear stress of short duration (1 or 3 h) reduces intercellular communication as a consequence of morphological changes (4, 39), loss and/or internalization of membrane-bound Cx43 (10, 27, 35, 36), and possibly also inhibited trafficking of newly synthesized Cx43 to the membrane. Our finding that plasmalemmal ZO-1 immunostaining decreased in a somewhat similar manner to Cx43 indicates that ZO-1 and Cx43 might interact in MC3T3-E1 and MLO-Y4 cells as has been reported for other cell types (16, 19, 28, 50). Because interaction with ZO-1 has been suggested to stabilize Cx43 at the cell surface (49), the downregulation of ZO-1 by shear stress might lead to redistribution of Cx43 to intracellular compartments.

Functional studies on junctional communication have indicated that during shear stress exposure, dye coupling is reduced as gap junction protein expression decreases on the appositional membranes. Our observation of impaired dye coupling during the early period of laminar shear stress in cultured bone cells was similar to that reported for endothelial cells (10). Hence, these results verify our conclusion that fluid shear stress disrupts cell-to-cell communication. Biochemical evidence for the redistribution of Cx43 due to fluid shear stress is provided by the observed decrease in Cx43 P2 from the membrane, which is believed to be the predominant form of this gap junction protein that forms functional gap junction channels (35, 36) and an increase in all three forms of Cx43 in the cytosol.

Our findings on the distribution of Cx43, intercellular coupling, and the phosphorylation level of Cx43 after the steady shear stress exposure in MLO-Y4 cells differ from a recent report (4), which states that migration of Cx43 from the perinuclear region toward the dendritic processes and intercellular coupling increased after MLO-Y4 cells were subjected to fluid flow. Although differences might have arisen from usage of different antibodies, the analytical methods applied, or the composition of culture media, the previous study noted a lack of correlation between Cx43 distribution and the enhanced intercellular coupling, which suggests that another connexin might upregulate cell-to-cell communication after shear stress exposure. We analyzed both Cx43 and Cx45 in our experiments, and our data suggest that although Cx43 is the major gap junction protein that regulates junctional communication in MC3T3-E1 and MLO-Y4 cells, Cx45 can be upregulated under high shear stress conditions. The previous study also analyzed various osteoblastic cells (2T3, ROS17/2.8, MC3T3-E1), reported no effect of
fluid flow on cell-to-cell communication, and concluded that osteoblasts are less responsive than osteocytes to such stimuli (see also Ref. 25). However, numerous previous studies on osteoblastic cells have clearly demonstrated that stress enhances the production of second messengers such as cAMP, NO, Ca^{2+}, and prostaglandin (18, 20, 24, 41, 45) and alters cell morphology including the reorganization of the actin cytoskeleton (39). Our data indicate that osteoblastic MC3T3-E1 cells do respond to fluid shear stress.

We have observed differential mRNA expression of Cx43, Cx45, and ZO-1 in response to different shear stress levels. Low shear stress upregulated Cx43 expression in both cell types, whereas high shear stress upregulated Cx45 expression in both cell types. Previous studies have shown that when Cx43 function is inhibited in avian osteogenic tissue, Cx45 is upregulated to fulfill at least some aspect of the missing functions (33). Such selective upregulation of Cx43 and Cx45 by different levels of shear stress provides evidence that patterns of gene expression are transduced by the mechanical stimulus that would be expected to qualitatively alter the junctional phenotype and may correspond to either differentiation or dedifferentiation of bone cells (13, 31, 33, 34, 42, 44).

Although the mechanotransduction cascade leading from shear stress to altered gene expression patterns remains to be fully elucidated, fluid shear stress has been shown to enhance second-messenger production in cultured bone cells. In bone cells, cAMP is regulated by prostaglandin, and both are increased under fluid-flow conditions (41); cAMP has been shown to upregulate both the mRNA and protein of Cx43 and Cx45 in cultured cardiac myocytes (8). Therefore, a possible mechanism by which cultured bone cells might respond to fluid flow would involve disruption of cell-to-cell communication and enhanced production of prostaglandins. This would elevate cAMP, which in turn would upregulate expression and phosphorylation of either Cx43 or Cx45 depending on the magnitude of the shear stress and the duration of the exposure period. Subsequently, cellular differentiation would be initiated, and disconnected cells or the remaining network would begin focal bone remodeling.

The decrease in ZO-1 mRNA and protein expression with increased levels of shear stress that we have observed may also play an important role in bone remodeling. Truncation mutants of the tight junction protein ZO-1 have been shown to disrupt epithelial cell morphology, which suggests that ZO-1 may be involved in the regulation of cellular differentiation (43). Moreover, downregulation of ZO-1 and occludin appear to be related to phenotypic changes associated with epithelial cell transformation (32). Therefore, it seems likely that ZO-1 may also be involved in mediating cell differentiation in cultured bone cells.

The results described here were obtained under in vitro conditions in tissue culture. In vivo, there is increasing evidence that osteocyte cell processes are surrounded by the pericellular matrix with transverse tethering filaments (55), whereas in culture, there is no encircling support structure. In addition, the recent theoretical model by You et al. (60) brings to our attention that in vivo, the fluid shear stresses on the cell body are much smaller than those on the membrane in cell processes, so that the fluid drag force on the transverse filaments in the pericellular matrix is much greater than the fluid shear force on the cell-process membrane. Therefore, it is possible that under in vitro conditions the actin cytoskeleton in the cell body responds to the fluid shear stress rather than the more rigid actin bundle in the cell process. However, You et al. (60) predicted that the fluid drag on the tethering filaments can produce a 20- to 100-fold amplification in the strain on the actin filament bundle in the cell process. This amplification is sufficient to elicit intracellular signaling responses in all cell cultures with deformed substrates.

In summary, we have shown that fluid-induced shear stress is an important biophysical signal in bone mechanotransduction. Our observations suggest that in both osteoblastic and osteocyte cell lines, fluid shear stress of the magnitude expected to occur in bone tissue disrupts junctional communication, rearranges junctional proteins, and determines de novo synthesis of specific connexins to an extent that depends on the magnitude of the shear stress. Such disconnection from the bone cell network due to the fluid shear stress may provide part of the signal whereby the disconnected cells or the remaining network initiates focal bone remodeling.

We thank Marcia Urban for technical assistance, Dr. Wei Li for advice on separating membrane and cytosolic Cx43, Dr. Karen Cusato for advice on use of the Live/Dead cell assay, Dr. Elliot L. Hertzberg (Albert Einstein College of Medicine) and Dr. Thomas H. Steinberg (Washington University School of Medicine) for generous supply of Cx43 and Cx45 antibodies, and Dr. Kenneth J. McLeod (State University of New York, Stony Brook) and Dr. Lynda F. Bonewald (University of Texas Health Science Center) for graciously providing the MC3T3-E1 and MLO-Y4 cell lines.

This work was supported primarily by National Institutes of Health (NIH) Grant HL-19454 (principal investigator, S. Weinbaum) and a Gilleece Fellowship (City University of New York Graduate School) with additional support provided by NIH Grants DK-41918 and NS-34891 (principal investigator, D. C. Spray).

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