Inhibition of Gap-Junctional Communication Induces the Trans-differentiation of Osteoblasts to an Adipocytic Phenotype in Vitro*

Osteoblasts and adipocytes are thought to differentiate from a common stromal progenitor cell. These two phenotypically mature cell types show a high degree of plasticity, which can be observed when cells are grown under specific culture conditions. Gap junctions are abundant among osteoblastic cells in vivo and in vitro, whereas they are down-regulated during adipogenesis. Gap junctional communication (GJC) modulates the expression of genes associated with the mature osteoblastic phenotype. Inhibition of GJC utilizing 18α-glycyrrhetinic acid (AGRA) blocks the maturation of pre-osteoblastic cells in vitro. Moreover, cytoplasmic lipid droplets are detectable at the end of the culture period, suggesting that GJC inhibition may favor an adipocytic phenotype. We used several human osteoblastic cell lines, as well as bone-derived primary osteoblastic cells, to show that confluent cultures of human osteoblastic cells grown under osteogenic conditions developed an adipocytic phenotype after 3 days of complete inhibition of GJC using AGRA or oleamide, two dissimilar non-toxic reversible inhibitors. Development of an adipogenic phenotype was confirmed by the accumulation of triglyceride droplets and the increase in mRNA expression of the adipogenic markers perilipin, lipoprotein lipase, and α-glycerophosphate dehydrogenase. Glycyrrhizin acid, a noninhibitory AGRA analog, or α-bromopalmitate, a nondegradable fatty acid, had no effect. Modulation of skeletal GJC may represent a new pharmacological target by which inhibition of marrow adipogenesis can take place with the parallel enhancement of osteoblastogenesis, thus providing a novel therapeutic approach to the treatment of human age-related osteopenic diseases and postmenopausal osteoporosis.

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Osteoblasts and adipocytes are phenotypically distinct mature cells thought to differentiate from a common mesenchymal progenitor cell (1–2). The molecular events that determine lineage progression toward a specific phenotype remain to be fully characterized. Either cell type can be cultured in vitro under conditions that foster or maintain their respective phenotypes. Osteogenic conditions include the presence of ascorbic acid, β-glycerophosphate, and dexamethasone or vitamin D3, depending on the maturational stage of the osteoblast precursor, in the culture medium. In contrast, adipogenic conditions include the presence of 3-isobutyl-1-methylxanthine (IBMX), insulin, and dexamethasone in the culture medium. Nutall et al. (3) have shown that mature human osteoblastic cells are capable of transdifferentiation to adipocytes when cultured in the presence of dexamethasone and IBMX. On the other hand, Park et al. (4) have shown that cloned single human adipocytes can dedifferentiate into fibroblast-like cells, which subsequently differentiate into osteoblasts or adipocytes under appropriate culture conditions. This plasticity between the differentiation of osteogenic and adiogenic human cells provides further support for a common precursor and has significant physiological implications, because the decrease in bone mass associated with age-related osteopenia and osteoporosis is accompanied by an increase in bone marrow adipose tissue (5–6).

Gap junctions (GJ) are aggregations of individual gated cell-to-cell channels that allow the exchange of small molecules (up to 1 kDa) between the cytoplasms of adjacent cells (7). Connexin43 (Cx43) has been identified as the major component of gap junctions in osteoblastic cells (8–11) and in bone (12). Cx43 expression and junctional permeability increase during in vitro osteoblastic maturation and are regulated by osteotropic factors (8, 13, 14). The responsiveness of osteoblastic cells to stimulation by parathyroid hormone is modulated by gap junctional communication (GJC) (15, 16). Moreover, inhibition of gap junctional communication interferes with the maturation process of osteoblastic cells in culture (17, 18). In contrast, murine marrow-derived preadipocytic cells undergoing in vitro adipogenic differentiation down-regulate Cx43 and GJC (19). Accordingly, these data suggest that GJC may play a critical role in modulating the progression of progenitor cells toward a specific phenotype in vivo. Moreover, we have reported that in the rat, Cx43 is down-regulated as a function of age in most tissues examined (20).

In this study we investigate whether modulation of GJC per

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* This work was supported in part by the Veterans Affairs Research Service and GRECC. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: IBMX, 3-isobutyl-1-methylxanthine; GJ, gap junction; GJC, GJ communication; FBS, fetal bovine serum; bp, base pair(s); RT-PCR, reverse transcriptase-polymerase chain reaction; LPL, lipoprotein lipase; GA, glycyrrhizinic acid; PPAR, peroxisome proliferator-activated receptor; AGRA, 18α-glycyrrhetinic acid; EF, elongation factor.
GJC Modulates the Plasticity of Osteoblasts to Adipocytes

It was found that the adjacent cell transferred calcine to a subsequent adjacent cell in a direction away from the donor cell, as when transfer was to yet a third cell away from the donor, and so on. Dye transfer to 5 or more cells away from the donor was difficult to quantify and was scored as 5. GJC was scored only when the donor was a single cell at least 10 cells away from another single donor cell.

**Isolation and Analysis of RNA**—For RNA isolation, cells were grown in 60-mm or 100-mm dishes (Costar). At the indicated times, the medium was removed, and total RNA was extracted as previously described (15) using the Trizol Reagent (Life Technologies, Inc., Manassas, VA) according to the manufacturer’s instructions. RNA was quantitated spectrophotometrically. For Northern blot analysis, 10 to 20 mg of RNA was probed previously described (13). A mouse bone sialoprotein (BSP) cDNA probe was kindly provided by Dr. Marian Young, the mouse osteocalcin (OC) probe was a 371-bp fragment obtained by RT-PCR corresponding to positions 8–379 of GenBank™/EBI locus mmMGBPR, and the mouse GAPDH fragment was from Ambion (Austin, TX).

For quantitative RT-PCR analysis, total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI). RNA was precipitated in 2 M LiCl (Sigma) solution, and quantitated spectrophotometrically. Five micrograms of high molecular weight RNA was reverse transcribed using MuLV reverse transcriptase, 200 pmol random hexamer primer, and 50 pmol of oligo(dT) (16). Aliquots (4%) of the total cDNA were amplified in each PCR in a 20-μl reaction mixture containing 10 pmol of each primer in a standard PCR buffer supplemented with 0.5 μCi of [32P]dCTP (10 μCi/μl; PerkinElmer Life Sciences). Each sample was run in duplicate. Controls lacking reverse transcriptase were treated in the same fashion. All PCR reagents were from Perkin-Elmer (Norwalk, CT). Amplifications were performed in a GeneAmp9600 thermal cycler (Perkin-Elmer) for 17–28 cycles (typically: 94 °C for 30 s; 55 °C for 60 s; 72 °C for 60 s) after an initial denaturation of 94 °C for 2 min. PCR products in a 10-μl aliquot were size separated by electrophoresis in 6% acrylamide/Tris-borate-EDTA gels. Quantitation of the amplification reactions was performed by electronic autoradiography using an InstantImager analyzer (Packard Instrument Co., Meriden, CT). Semi-quantitative analysis of gene expression was assessed normalized to the corresponding elongation factor (EF-1α) PCR product. Primer Sequences: mouse osteocalcin (371-bp product: GenBank™/EBI locus mmMGBPR), mOFC (5'-CAAGTCCAACACAGCAGGTT'-3') and mOC-R (5'-AAAGGCCGACCTGCCAGATT-3'); human PPAR-γ (419-bp product: HU7921012; hPPAR-F (5'-ATTTCCCTATGCGCCAGAGG-3') and hPPAR-R (5'-AGCTTTATTCTGCACAGACAGAATG-3'); human LPL (276-bp product; HSLPLF: hLPL-F (5'-GAGATTCTCTGTGAGTCATGCACC-3') and hLPL-R (5'-CTCGCAATAAGAGACATCTTCT-3'); human EF-1α (215-bp product; HUMPT114a: hEF1a-F (5'-AGGTGATATCCTGGAACATCC-3') and hEF1α-R (5'-AAAGTGTGATAGTCTCAGAACG-3').

**Cytochemistry**—Cells were washed briefly in phosphate-buffered saline and fixed in 2% formaldehyde, 0.2% glutaraldehyde for 30 min at 4 °C. Lipid droplets were stained using a 3% solution of Sudan IV (Sigma) or Oil-Red-O (Sigma) in isopropyl alcohol/water (3:2) according to the manufacturer’s specifications. Matrix-bound mineral content was determined using alizarin red-S as previously described (27).

**Quantitation of Cytoplasmic Triglycerides**—Cellular lipids were extracted directly from the tissue culture wells with hexane/isopropanol alcohol (3:2; v/v) as described (28). Cellular cholesterol and triglyceride were measured by published methods (29, 30). After the lipid extractions, the proteins were dissolved in 0.1 M NaOH, and protein concentration was determined by the method of Lowry et al. (31) using bovine serum albumin as standard. Cholesterol and triglyceride mass were expressed as μg lipid per mg of cell protein.

**Statistical Analysis**—Data are expressed as the mean ± S.E. for each group. Statistical differences among treatment groups were evaluated with analysis of variance. p values less than 0.05 were considered to be significant.

**RESULTS**

**Effect of AGRA on GJC during Long-Term Cultures of MC3T3-E1 Cells**—MC3T3-E1 cells were seeded as described under “Experimental Procedures,” and the following day (day 0) the medium was replaced with medium supplemented with ascorbic acid and β-glycerophosphate (osteogenic medium). Under these conditions, these preosteoblastic cells undergo a maturation process similar to that of primary osteoblastic cells.
Parallel cultures were grown in osteogenic medium in the presence or absence of 100 \( \mu \text{M} \) AGRA, 100 \( \mu \text{M} \) GA, or vehicle (Me\(_2\)SO) for 3 to 28 days. At this concentration, AGRA had no detectable deleterious effect (i.e., no decrease in cell number) in these cells (not shown). GJC was measured from 2 to 6 h after overlaying the donor cells on those days in which the medium was changed. GJC increased with time in culture in cells treated with either vehicle or 100 \( \mu \text{M} \) GA in a fashion similar to untreated cells at all times tested. In contrast, AGRA inhibited GJC within the time frame in which it was assayed (Table I).

**Effect of Inhibition of GJC on the Expression of Osteoblastic Markers in MC3T3-E1 Cells—**MC3T3-E1 cells were seeded and grown as described under “Experimental Procedures.” Parallel cultures were grown in the presence or absence of vehicle (Me\(_2\)SO), 100 \( \mu \text{M} \) GA, or 100 \( \mu \text{M} \) AGRA and used to determine matrix-bound mineral or for RNA isolation. Growth medium was changed every 3–4 days, and fresh test substances were added every time the medium was changed. AGRA-mediated inhibition of GJC resulted in a decrease in matrix mineral content at all times tested (Table I). Northern blot analysis demonstrated that expression of BSP and OC (Table I) mRNAs, assayed within 4 h after addition of chemicals, were decreased only when GJC was inhibited. Semiquantitative RT-PCR analysis of the same RNA used in the Northern blot experiments confirmed the Northern blot data (not shown).

Because inhibition of GJC in long-term cultures of MC3T3-E1 preosteoblastic cells inhibited the development of the osteoblast phenotype, we investigated whether it would have an effect on the appearance of the adipocytic phenotype. Sudan IV staining of cells grown under osteogenic conditions for 28 days showed that lipid droplets accumulated in the cytoplasm of some of the cells only when GJC had been inhibited by AGRA (Fig. 1A).

**Effect of AGRA, Oleamide, and GA on GJC in Human Osteoblastic Cells—**Having found that inhibition of GJC not only inhibited the development of an osteoblastic phenotype but stimulated adipocytic characteristics (cytoplasmic lipid droplets) in long-term cultures of murine preosteoblastic MC3T3-E1 cells, we wanted to determine whether inhibition of GJC for a relatively short term had an effect on the capacity of phenotypically mature human osteoblastic cells to develop an adipocytic phenotype. Human U-2 OS and bone-derived primary osteoblastic (hOB) cells used in these studies showed alkaline phosphatase activity and expressed bone sialoprotein mRNA (not shown). Cells were seeded and grown under osteogenic conditions in parallel cultures for 4, 8, or 15 days as described under “Experimental Procedures.” In each case the cells were exposed to the GJC inhibitors AGRA or oleamide, the noninhibitory analog GA, or vehicle for 3 consecutive days. Because AGRA and oleamide were effective in inhibiting GJC for approximately a 24-h period, the medium was replenished with fresh osteogenic medium (containing vehicle, AGRA, oleamide, or GA) each consecutive day. On the fourth day after beginning the treatments with the chemical agents, the cells were fixed and stained for cytoplasmic lipid droplets with Sudan IV or Oil-Red-O. Lipid staining showed the accumulation of cytoplasmic droplets only in cells from cultures grown under osteogenic conditions for 8 days (confluent cultures) after GJC was inhibited by AGRA or oleamide for 3 consecutive days (Fig. 3). No accumulation of lipid droplets was detected in sparse U-2 OS cultures grown for 4 days or in compact overconfluent cultures grown for 15 days in which GJC had been inhibited with either AGRA or oleamide for an additional 3 consecutive days (not shown).

**Effect of Inhibition of GJC on the Expression of Adipocytic Markers in Human Osteoblastic Cells—**Human U-2 OS cells express phenotypic characteristics of mature osteoblasts (33). Also the bone-derived primary osteoblastic (hOB) cells used in these studies showed alkaline phosphatase activity and expressed bone sialoprotein mRNA (not shown). Cells were seeded and grown under osteogenic conditions in parallel cultures for 4, 8, or 15 days as described under “Experimental Procedures.” In each case the cells were exposed to the GJC inhibitors AGRA or oleamide, the noninhibitory analog GA, or vehicle for 3 consecutive days. Because AGRA and oleamide were effective in inhibiting GJC for approximately a 24-h period, the medium was replenished with fresh osteogenic medium (containing vehicle, AGRA, oleamide, or GA) each consecutive day. On the fourth day after beginning the treatments with the chemical agents, the cells were fixed and stained for cytoplasmic lipid droplets with Sudan IV or Oil-Red-O. Lipid staining showed the accumulation of cytoplasmic droplets only in cells from cultures grown under osteogenic conditions for 8 days (confluent cultures) after GJC was inhibited by AGRA or oleamide for 3 consecutive days (Fig. 3). No accumulation of lipid droplets was detected in sparse U-2 OS cultures grown for 4 days or in compact overconfluent cultures grown for 15 days in which GJC had been inhibited with either AGRA or oleamide for an additional 3 consecutive days (not shown).

### Table I

| Treatment   | GJC* | Mineralization* | BSP mRNA* | OC mRNA* |
|-------------|------|-----------------|-----------|----------|
| Vehicle     | ≥5   | 197 ± 18.5      | 100       | 100      |
| 100 \( \mu \text{M} \) GA, 100 \( \mu \text{M} \) AGRA | ≥5   | 195 ± 19        | 98 ± 16   | 96.7 ± 0.9 |
| 100 \( \mu \text{M} \) AGRA | 0.12 ± 0.21 | 21 ± 5.1        | 5 ± 0.6   | 18.9 ± 1.9 |

* GJC was assayed in cells grown for 7, 14, 21, and 28 days as described under “Experimental Procedures.” 100 \( \mu \text{M} \) AGRA inhibited GJC at all times tested. The results shown represent the data obtained on cells cultured for 28 days.

* Mineralization was assessed as the amount of alizarin red-S bound per unit area (\( \mu \text{g/cm}^2 \)) on cells cultured for 28 days as previously described (25).

* BSP mRNA levels were determined by Northern blot analysis and quantitated by densitometric analysis as described. Values represent percentages of maximal expression (vehicle treatment defined as 100%) of RNA isolated from 28-day cultures.

* OC mRNA levels were determined by Northern blot analysis and RT-PCR as described. Values represent percentages of maximal expression (vehicle treatment defined as 100%), from RT-PCR data, of RNA isolated from 28-day cultures. Northern blot analysis showed a more dramatic inhibitory effect with AGRA treatment.
conditions described. Treatment with a (under “Experimental Procedures.” hOB cells were treated with vehicle as a result of AGRA or oleamide treatment. Cytoplasmic lipid droplets were detected only when GJC was inhibited as shown). No Tx ( ), untreated cells; Veh ( ), vehicle; GA20 (+), 20 μM GA; GA90 (×), 90 μM GA; AGRA20 (▲), 20 μM AGRA; AGRA70 (▼), 70 μM AGRA; AGRA90 (▲), 90 μM AGRA. Oleamide treatment is represented as follows: in U-2 OS cells (top), large triangles, 50 μM oleamide; and inverted large triangles, 70 μM oleamide; in hOB cells (bottom), large triangles, 70 μM oleamide; and large inverted triangles, 90 μM oleamide. Each point represents the mean ± S.E. of n ≥ 20.

Analysis of the lipid content in U-2 OS cells indicated that inhibition of GJC resulted in a 3–5-fold increase in triglyceride levels in the cytoplasm of the cells (Fig. 5), whereas the cholesterol levels were not affected (not shown). In a similar fashion, analysis of lipid content in hOB cells showed that inhibition of GJC resulted in a 3–8-fold increase in cytoplasmic triglyceride content without affecting cholesterol levels (not shown).

To further characterize the effect of inhibition of GJC on the development of an adipogenic phenotype by human osteoblastic cells, the level of expression of PPAR-γ2, LPL, and EF-1α transcripts was evaluated using semiquantitative RT-PCR in U-2 OS cells. Inhibition of GJC with AGRA resulted in an increase in the expression of the adipogenic-specific genes PPAR-γ2 and LPL, whereas expression of EF-1α was unaffected (Fig. 6A). Similar results were obtained in U-2 OS cells treated with oleamide in hOB cells where GJC had been inhibited with either AGRA or oleamide (not shown).

DISCUSSION

The results demonstrate that the effects of AGRA-mediated inhibition of GJC inMC3T3-E1 osteoblastic cells are very similar to those obtained as a result of gene-mediated inhibition of GJC utilizing dominant-negative mutants of Cx43 (18). AGRA-mediated inhibition of GJC has been utilized as a means to demonstrate other biological events in which GJC was found to play a significant role (34–36). We have previously reported that Cx43 (13) and GJC (16) increase during in vitro osteogenesis. Inhibition of GJC in MC3T3-E1 cells with AGRA is reversible, and because the medium containing the inhibitor is replaced every 3–4 days, it is likely that GJC is at least partially recovered among the cells before the addition of fresh inhibitor blocks it again. Long-term GJC inhibition was sufficient to interfere with the ability of osteoblastic cells to express two genes, BSP and OC, intimately associated with the mature osteoblastic phenotype. GJC inhibition also profoundly affected the capacity of the osteoblastic cells to produce a mineralized extracellular matrix, the ultimate phenotypic hallmark of an osteoblast. In addition, GJC inhibition not only blocked the progression of preosteoblastic cells toward the mature osteo-

FIG. 4. Sudan IV staining of hOB2 (left), HOS (center), and SaOS (right) cells exposed to noninhibitors of GJC (top) or to GJC inhibitors (bottom) at the indicated concentrations.

FIG. 2. GJC in U-2 OS (top) and hOB (bottom) cells. GJC was assayed as described under “Experimental Procedures” in untreated cells or cells that had been exposed to different treatments as a function of time. Both cell types were very well coupled, with hOB cells showing a somewhat higher basal level. Treatments were as follows: No Tx (■), untreated cells; Veh (○), vehicle; GA20 (+), 20 μM GA; GA90 (×), 90 μM GA; AGRA20 (▲), 20 μM AGRA; AGRA70 (▼), 70 μM AGRA; AGRA90 (▲), 90 μM AGRA. Oleamide treatment is represented as follows: in U-2 OS cells (top), large triangles, 50 μM oleamide; and inverted large triangles, 70 μM oleamide; in hOB cells (bottom), large triangles, 70 μM oleamide; and large inverted triangles, 90 μM oleamide. Each point represents the mean ± S.E. of n ≥ 20.

FIG. 3. Sudan IV staining of U-2 OS (top) and Oil-Red-O staining of hOB (bottom) cells. U-2 OS cells were treated with vehicle (left), 90 μM GA (center), or 90 μM AGRA (right) for 3 days as described under “Experimental Procedures.” hOB cells were treated with vehicle (left), 90 μM α-bromopalmitate (center), or 90 μM oleamide (right). Cytoplasmic lipid droplets were detected only when GJC was inhibited as a result of AGRA or oleamide treatment.

To ascertain that lipid droplet accumulation in the cytoplasm of AGRA-treated cells was caused by inhibition of GJC and not uptake of excess lipids in the growth medium or fatty acid-induced adipogenesis, U-2 OS and hOB cells were grown for 7 days and exposed to the nonmetabolizable fatty acid α-bromopalmitate (90 μM) from day 8 to day 11 under the culture conditions described. Treatment with α-bromopalmitate did not induce accumulation of cytoplasmic lipid droplets (Fig. 3).

To confirm that the observed results were not an isolated phenomenon and that indeed inhibition of GJC could promote the trans-differentiation of other human osteoblastic cells to an adipocytic phenotype, the osteoblastic cell lines SaOS, HOS, and an additional primary osteoblastic culture were exposed to the same treatment. In all cases the osteoblastic cells accumulated lipid droplets only when complete inhibition of GJC by either AGRA or oleamide was achieved (Fig. 4). AGRA- and oleamide-mediated inhibition of GJC in SaOS, HOS, and additional primary osteoblastic cultures was time- and dose-dependent in a fashion similar to that observed for U-2 OS (not shown).

The results demonstrate that the effects of AGRA-mediated inhibition of GJC in MC3T3-E1 osteoblastic cells are very similar to those obtained as a result of gene-mediated inhibition of GJC utilizing dominant-negative mutants of Cx43 (18). AGRA-mediated inhibition of GJC has been utilized as a means to demonstrate other biological events in which GJC was found to play a significant role (34–36). We have previously reported that Cx43 (13) and GJC (16) increase during in vitro osteogenesis. Inhibition of GJC in MC3T3-E1 cells with AGRA is reversible, and because the medium containing the inhibitor is replaced every 3–4 days, it is likely that GJC is at least partially recovered among the cells before the addition of fresh inhibitor blocks it again. Long-term GJC inhibition was sufficient to interfere with the ability of osteoblastic cells to express two genes, BSP and OC, intimately associated with the mature osteoblastic phenotype. GJC inhibition also profoundly affected the capacity of the osteoblastic cells to produce a mineralized extracellular matrix, the ultimate phenotypic hallmark of an osteoblast. In addition, GJC inhibition not only blocked the progression of preosteoblastic cells toward the mature osteo-
Fig. 5. Changes in triglyceride content in the cytoplasms of U-2 OS cells as a result of inhibition of GJC; Co, untreated; Veh, MeSO4; GA, 90 μM GA; Palm, 90 μM α-bromopalmitate; AGRA, 70 μM AGRA; Ole, 70 μM oleamide-treated cells.

Fig. 6. Changes in the expression of adipocytic gene markers in U-2 OS cells as a result of inhibition of GJC. A, RT-PCR analysis of the expression of PPARγ-2, LPL, and EF-1α mRNAs in U-2 OS cells treated with 90 μM GA or 70 μM AGRA for 3 days as described under “Experimental Procedures.” Total RNA was treated in the presence of reverse transcriptase (+RT) or in its absence (−RT). The products of these reactions were amplified by PCR using specific primers for 23 cycles to detect EF-1α transcripts or for 27 cycles to detect PPARγ-2 and LPL transcripts. Inhibition of GJC by 70 μM AGRA increased the expression of PPARγ-2 (419-bp product) and LPL (276-bp product) transcripts whereas it had no effect on EF-1α (235-bp product) transcription. B, relative level of expression of PPARγ-2 and LPL genes as a function of treatment, determined from the actual number of dpm obtained for each amplified band normalized to the counts obtained for EF-1α. Mean ± S.E. of three independent experiments.

Our results also demonstrate that cultured human bone-derived osteoblasts, as well as widely used osteoblastic cell lines, undergo trans-differentiation toward the adipogenic phenotype after 3 days of AGRA- or oleamide-mediated inhibition of GJC despite being grown under osteogenic culture conditions. Adipogenesis was characterized by the expression of functional as well as molecular markers. Lipid staining and subsequent analysis showed that indeed triglycerides accumulate in the cytoplasm of cells as a consequence of GJC inhibition by either AGRA or oleamide. This phenotypic change took place with a parallel increase in PPARγ2 and LPL transcripts, molecular markers of adipogenesis. In the studies described we use AGRA, a polycyclical lipid, as well as oleamide, an amide of oleic acid, as inhibitors of GJC. It has previously been reported that certain lipids, in particular fatty acids, are capable of inducing differentiation of preadipocytes to adipocytes (37). However, we have found no evidence that lipids with a chemical structure resembling that of AGRA or oleamide are capable of inducing adipogenesis. As a control we have used GA, a glycoside of 18-β-glycyrrhetinic acid (21), which does not inhibit GJC, at the same concentration and found to have no effect on adipogenesis. 18-β-glycyrrhetinic acid also inhibited GJC in osteoblastic cells; however, in our hands it had a significantly higher toxicity than its α-isomer and was not used for that reason (data not shown). To further exclude the possibility that the GJC inhibitors may be inducing adipogenesis as a consequence of their lipid nature, we used the nonmetabolizable fatty acid α-bromopalmitate and found that at concentrations of up to 90 μM, it also was unable to induce trans-differentiation of human bone-derived osteoblastic cells to adipocytes, suggesting that lipids alone are not sufficient to induce this process in these cells. Similarly, Nutall et al. (3) reported that α-bromopalmitate by itself was unable to induce expression of an adipocytic phenotype in treated osteoblasts.

There is a possibility that AGRA may be inducing adipogenesis via a mechanism different from inhibition of GJC. Previous studies have shown that AGRA can inhibit calcium waves in addition to inhibiting GJC (38). We have used oleamide, which does not inhibit intercellular calcium signaling (38), instead of AGRA and found it to inhibit GJC in a dose-dependent fashion and to have similar effects in inducing trans-differentiation to adipocytes. We observed that complete inhibition of GJC with either AGRA or oleamide was required in order for osteoblastic cells to develop adipocytic characteristics within the time frame described. The results obtained using AGRA and oleamide, two inhibitors with different chemical structures, support a role for GJC in determining lineage progression of skeletal cells with multipotential capacity. A future direction is to determine the adipogenic potential of marrow-derived progenitor cells from Cx43-null mice, whose osteoblastic cells were reported to have a decreased osteogenic potential (39). Exposures of U-2 OS cell cultures grown for 4 days (sparsely populated) or for 15 days (tightly compact) to the same GJC inhibitory conditions (3 days) did not increase cytoplasmic lipid content. These results suggest that there may be a window of time, during the culture of these osteoblastic cells, during which inhibition of GJC will lead to adipogenesis. Genetic approaches using inducible expression vectors which will profoundly inhibit GJC, such as dominant-negative for Cx43 and Cx45, now under development, will confirm the results obtained with the chemical inhibitors.

Our results support the concept that plasticity exists among cells of the stromal lineage and that modulation of GJC may be a mechanism involved in determining progression toward an osteoblastic or adipocytic fully differentiated phenotype. Whereas cell-to-cell coupling via gap junctions would favor osteogenesis, uncoupling would favor adipogenesis. Changes in GJC could contribute to a shift in lineage progression by affecting the second-messenger systems activated in response to external stimuli or the rate of transcription of specific genes associated with the fully differentiated phenotypes. In support of this concept, Vander Molen et al. (15) have demonstrated that the responsiveness of osteoblasts to parathyroid hormone changes as a function of GJC, and Lecanda et al. (40) have identified specific regulatory sequences in the osteocalcin promoter which are sensitive to GJC in osteoblastic cells.

Cells coupled via gap junctions may transfer second-messenger molecules, such as calcium, inositol phosphates, or cyclic nucleotides, from the stimulated cell to neighboring cells. Alternatively, when uncoupled, the relative concentration of the second messenger, e.g., calcium, could reach higher intracellular levels than when the cell is coupled. Thus cell coupling may influence the amplitude and the duration of the response to a stimulus, which may, in turn, regulate the cascade of events (e.g., protein kinases) leading to transcription factor activity modulation. Inactivation of a particular set of transcription factors, such as those involved in the regulation of OC or BSP...
expression, in parallel with activation of a different set, such as PPARγ2 or others involved in adipogenesis, may cause a geno-
typic switch leading to a change in the phenotype of a cell. The
role of GJC in this lineage interrelationship may have impor-
tant consequences in terms of the progression of osteoporo-
sic diseases. The excess adipogenesis in postmenopausal women
and in age-related osteopenia may occur at the expense of
osteogenesis and may involve a decrease in GJC as a result of
aging or endocrine changes. We have recently demonstrated an
age-related decrease in marrow osteoblastogenesis in humans
(25). Moreover, in the femurs of young mice there are twice as
many GJ (among osteoblasts, stromal, and hematopoietic cells)
than in those from older animals (41).

Modulation of skeletal GJC may represent a pharmaco-
tological target by which inhibition of marrow adipogenesis can take
place with the parallel enhancement of osteoblastogenesis. Such a
scenario could provide a novel therapeutic approach to the
treatment of human age-related osteopenic diseases and
postmenopausal osteoporosis.

Acknowledgments—We thank David Vazquez and Nubia Rodriguez
for their expert technical assistance. We also thank Dr. Marian F.
Young (National Institutes of Health) for the plasmid mBSP1.

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