Bone morphogenetic proteins (BMPs) occupy important roles during development serving to direct cells through specific differentiation programs. While several BMPs are essential for embryonic viability, their significance in mediating intercellular communication in the context of adult organ systems remains largely unknown. In the adult rat we characterized the tissue- and cell-specific transcription and translation of BMP-9. Utilizing a ribonuclease protection assay, we determined that in the adult animal, BMP-9 expression occurs predominantly in the liver. Furthermore, we determined that the non-parenchymal cells of the liver, i.e. endothelial, Kupffer, and stellate cells, are the major sources of this message. Western analyses corroborate the ribonuclease protection assay results, confirming that LEC and KC contain an abundance of immunoreactive BMP-9. Using \([^{125}\text{I}]\text{BMP-9}\), a receptor with specific binding affinity for BMP-9 was characterized in primary cultures of hepatic endothelial cells and Kupffer cells. BMP-9 binding to these cell types was observed to be fully reversible and highly specific for this ligand. Additionally, we demonstrate that BMP-9 is specifically internalized upon binding to its receptor. This may represent a novel BMP receptor and is the first to be characterized in primary cultures of mature liver non-parenchymal cells. Our results depict BMP-9 as a potential autocrine/paracrine mediator in the hepatic reticuloendothelial system.

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The secreted proteins of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily occupy central roles in cellular differentiation and growth. Members of this cytokine superfamily represent a highly conserved set of signaling proteins whose signaling in mature soft tissue systems remains largely unexplored. It is likely that the description of BMPs as differentiation factors is incomplete since most adult tissues consist of terminally differentiated cell populations. On the other hand, it is known that most cell types undergo constitutive turnover, the significance of which may not be fully appreciated beyond tissue damage-repair episodes.

With more than 20 mammalian BMPs now identified, only three type I receptors and three type II receptors have been cloned in mammals which have been shown to bind BMPs (11). The current receptor binding model as described for BMPs and TGF-\(\beta\) has been recently reviewed (12) and stipulates that BMP ligands bind type I and type II receptors with similar affinity. However, signal transduction requires both types of receptors. BMP ligands serve to bring type I and II receptors together. This allows the Ser/Thr kinase activity of the type II receptor to phosphorylate and activate the type I Ser/Thr kinase. The activated type I receptor then initiates intracellular signaling by phosphorylating cytoplasmic substrates known as SMAD proteins.

The receptor binding specificity of five BMPs have been described in the literature and indicates that there is some promiscuity exhibited by the known receptors for these ligands.
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(13, 14). There is relatively little published data describing cell-specific receptor binding affinities and cell-specific receptor populations. There are a number of publications documenting cross-linking of BMP ligands with their receptors (13) and a select few reports characterizing the intrinsic properties of BMP binding to both cell lines and primary cultures (15, 16).

We have investigated the tissue- and cell-specific expression of bone morphogenetic protein-9 (BMP-9). BMP-9 was originally cloned from a fetal mouse liver cDNA library and was shown to bind to specific receptors on HepG2 cells (17). In addition, BMP-9 was shown to cause a modest increase in proliferation of primary cultures of rat hepatocytes (17). These findings prompted us to investigate the endogenous sources of this cytokine and the cellular location of its receptor in livers of adult rats.

This report describes the cellular expression and receptor binding characteristics of bone morphogenetic protein-9. A survey of major organs shows that in the adult rat, BMP-9 message predominantly occurs in the liver. Within the liver, nonparenchymal cells, namely, Kupffer cells (KC), hepatic stellate cells, and liver endothelial cells (LEC) were used. In addition, the BMP-9 binding properties of both LEC and KC were characterized. Our findings indicate that BMP-9 signals are initiated via autocrine and/or paracrine mechanisms within the hepatic sinusoid. To our knowledge, this is the first report of a BMP receptor in the reticuloendothelial system of the liver.

EXPERIMENTAL PROCEDURES

Materials—All reagents were biomedical research grade. Collagenase (Type IV from Clostridium histolyticum), protease (Type XIV from Streptomyces griseus) (Sigma), metrizamide (Accurate Chemical & Scientific Corp., Westbury, NY) and Nycodenz (Sigma) for LEC and KC isolation by density gradient centrifugation. Unlabeled BMP-2, -4, -6, -7, -9, and -12 were provided by Genetics Institute Inc. (Cambridge, MA). BMP-2, -4, -6, and -12 were shown to possess biological activity by Genetics Institute, Inc. BMP-12 was positive in the MLB13MYC clone 14 assay (18). BMP-2, -4, and -6 gave positive results in the W-20-17 assay (19). BMP-9 was iodinated as described by Frolkis et al. (20) and shown to possess biological activity after iodination (17). TGF-β1, and TGF-β3 were obtained from (R&D Systems, Minneapolis, MN). The Pierce BCA protein assay (Pierce) was used to estimate total cell protein concentration from freshly isolated cell lysates prepared with cell lysis solution (1.25 mM HEPES, 62 mM sucrose, 0.25% Triton X-100, 6 mM deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 0.078 mM pepstatin A, 0.004 mg/ml leupeptin, 0.012 mg/ml aprotinin). Total cellular proteins were resolved by SDS-PAGE using a Tricine-based buffer system as described by Schagger and von Jagow (26). Proteins were transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) and subsequently immunoblotted using a BMP-9 monoclonal antibody raised in mouse kindly provided by Genetics Institute, Inc. Detection of the antigen-antibody complex was performed using the goat anti-mouse IgG (H + L)-HRP conjugate secondary antibody (Bio-Rad). BCA (Amersham Pharmacia Biotech). Membranes were exposed to Hyperfilm (Amersham Pharmacia Biotech) to obtain the desired intensity.

BMP-9 Binding, Cross-linking, and Binding Specificity—BMP-9 binding assays were performed on 18-h cultures of primary hepatocytes and liver endothelial cells and on 48-h primary cultures of Kupffer cells. Cells were incubated for 1 h at 37 °C in binding buffer (156.9 mM NaCl, 5.37 mM KCl, 1.26 mM CaCl₂, 0.64 mM MgSO₄, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.49 mM MgCl₂, 25 mM HEPES, 0.5% bovine serum albumin, pH 7.4) then transferred to binding buffer at 4 °C. Cells were exposed to 35–90 pM [125I]BMP-9 with appropriate concentrations of unlabeled BMP-9 for up to 24 h. After equilibration, cells were washed three times with ice-cold binding buffer then dissolved in cell solubilization buffer (25 mM HEPES, 10% glycerol, 1% Triton X-100, 1 mg/ml bovine serum albumin, pH 7.5) and counted. Cell numbers were determined from video images taken of the cells just prior to solubilization using a microscope-mounted video camera. Data were analyzed with Prism 2.0 software (Graphpad, Inc., San Diego, CA) and with the NIH program LIGAND.

The reversibility of [125I]BMP-9 binding was determined by displacing the radioligand with excess unlabeled BMP-9. LECs were incubated at 4 °C overnight with 77 pM [125I]BMP-9 alone or with 77 pM [125I]BMP-9 plus an additional 8-fold excess unlabeled BMP-9. Following the overnight incubation, the binding buffer was exchanged for buffer containing only 615 pM unlabeled BMP-9. At the appropriate time points, buffer was aspirated then the cells were washed, dissolved, and counted.

Endosomes were cross-linked to iodinated BMP-9 with 500 μM bisulfosuccinimidyl-suberate (BS₅). Cells were incubated overnight with 77 pM [125I]BMP-9 alone or in the presence of 77 μM or 1.9 mM unlabeled rhBMP-9. The cells were washed three times to remove unbound ligand. Bovine serum albumin-free binding buffer, with or without 500 μM BS₅, was then applied for 30 min at 4 °C. Cells were washed three times and solubilized in cell lysis solution. Twenty-five μg of cellular protein was resolved by 7.5% SDS-PAGE and the radioactive signal was captured on a PhosphorImager plate.

The specificity of the BMP-9-receptor binding interaction was determined by incubating KC and LEC with 77 pM [125I]BMP-9 at 4 °C overnight alone or with an additional 8- or 100-fold excess unlabeled competitor BMP. BMP-2, -4, -6, -7, and -12 were used in three separate experiments in 8-fold excess of the radioligand and in one experiment at 100-fold excess of the radioligand on both KC and LEC. BMP-7 was used in two experiments at 8-fold excess of radioligand on LEC. BMP-7 was used only once on KC at 8-fold excess and only once on KC at 100-fold excess (data not shown).

Receptor Internalization—After a 1-h incubation interval in serum-free medium, cells were washed three times with ice-cold serum-free medium to remove unbound ligand, then washed three times with ice-cold hypotonic acid wash solution (0.2 mM acetic acid, 0.5 mM NaCl, pH 2.5) (27). Cells were solubilized with cell lysis solution, acid wash-removable, and cell associated radioactivity were determined separately. Data points showing the internalization and cell surface radioactivity were determined separately. Data points showing the internalization and cell surface radioactivity were determined separately.
activity in the presence of 8-fold excess of unlabeled BMP-9 represent results from a single experiment. All data points with error bars represent pooled results from three independent experiments.

RESULTS

Total RNA was isolated from the major organs of healthy, mature Harlan Sprague-Dawley rats and probed for BMP-9 mRNA using a ribonuclease protection assay (RPA) (Fig. 1). The housekeeping gene cyclophilin was probed to indicate the equivalence of RNA loading as well as sample RNA integrity. Among the organs and tissues examined, it is clear that in the adult rat, BMP-9 transcription occurs predominantly in the liver.

The major component cell populations of the rat liver were isolated and the RPA was used to measure BMP-9 message levels (Fig. 2). Again, cyclophilin was probed to show equal RNA loading and sample RNA integrity. BMP-9 message was found to be expressed by KC, LEC, and hepatic stellate cells. Interestingly, we were unable to detect BMP-9 message RNA in liver parenchymal cells.

The presence of BMP-9 messenger RNA suggests that these cells are capable of translating BMP-9 protein. To confirm this assumption, Western blot analyses were performed on whole liver homogenate and LEC, KC, and hepatocyte cell lysates using a monoclonal antibody raised against recombinant human BMP-9 (Fig. 3). Freshly isolated cells were solubilized and samples of total cell proteins were resolved by SDS-PAGE under reducing conditions. As a control, 2 ng of rhBMP-9 was positioned in the first lane on the left and is indicated by the band at approximately 13 kDa, the expected molecular mass of monomeric BMP-9. Hepatocytes did not contain a detectable amount of the protein, however, BMP-9 is associated with KC and LEC following their isolation from the liver (Fig. 3, lanes LEC and KC). This monoclonal antibody clearly detects BMP-9 protein in samples of liver non-parenchymal cells (Fig. 3, lane NC). However, this method was not sensitive enough to detect BMP-9 protein in samples prepared from whole liver (Fig. 3, lane L). The immunoreactive bands with masses greater than 13 kDa are due entirely to secondary antibody immunoreactivity independent of the primary antibody (data not shown). The Western blot analysis corroborates the RPA results, establishing hepatic non-parenchymal cells as the primary source of BMP-9.

It is thought that BMPs act locally by autocrine and/or paracrine mechanisms. Upon finding that liver cells express BMP-9 we sought to identify a hepatic cell type capable of specifically binding this cytokine. Primary cultures of LEC and KC bound iodinated rhBMP-9 ([125I]BMP-9) as demonstrated by its displacement by unlabeled BMP-9 (Fig. 4, A and B). When cells were incubated at 4 °C in the presence of 35–90 pM [125I]BMP-9 and increasing concentrations of unlabeled, homologous competitor, the resulting competition binding isotherms depict a ligand-receptor interaction of high affinity. Our assay was unable to detect [125I]BMP-9 binding in primary cultures of hepatocytes (data not shown).

Binding parameters were similar between the two non-parenchymal cell types (Table I). LEC had an EC_{50} value of 57.2 pm with 95% confidence intervals ranging from 24.8 up to 131.9 pm. The EC_{50} value determined for KC was 5.1 pm with upper
and lower 95% confidence intervals at 7.1 and 3.6 pM, respectively. We then used the NIH program LIGAND to construct Scatchard plots and to estimate apparent $K_d$ and $B_{max}$ values, (Fig. 5, A and B).

Initially, we were unable to fit the combined Kupffer cell data using this program. Data for each experiment, when processed individually, yielded an extremely low apparent $K_d$ at 7.4 ± 11.5 attomolar (mean ± S.D., n = 6). Subsequently, we were able to fit the combined Kupffer cell data using a cooperativity setting of 20.1 which yielded an apparent $K_d$ of 24.9 pM and $B_{max}$ of 0.71 pM (Table I). In a 0.25-ml system, this represents 0.18 fmol/10^5 cells, or approximately 1100 receptors/cell. Visual inspection of Fig. 5A shows that specific binding is less than 1 fmol/well.

Combined data for experiments using liver endothelial cells yielded an apparent $K_d$ of 84.6 pM and $B_{max}$ of 2.41 pM (Table I). In a 0.25-ml system, this represents 0.63 fmol/10^5 cells, or approximately 3600 receptors/cell. Introducing a cooperativity factor of 0.1 made no significant difference to the fit of the liver endothelial cell data. Visual inspection of Fig. 5A shows that specific binding is approximately 1 fmol/well.

Scatchard analysis assumes that ligand binding is reversible. In order to confirm that this was occurring in our system LEC were incubated in the presence of 77 pM [125I]BMP-9 at 4 °C for increasing amounts of time after which the amount of ligand bound was determined (Fig. 5C). It was shown that [125I]BMP-9 binding by LEC increased over the 6-h interval and that unlabeled BMP-9, when added in excess, was able to displace the radioligand (Fig. 5D).

We performed affinity label cross-linking experiments with the radioligand. Binding equilibrium was established with primary cultures of KC and the primary amine-targeting cross-linking reagent BS3 was applied. The cellular proteins were then solubilized and resolved by SDS-PAGE under reducing conditions. The radioactive signal was imaged with a Phosphor-Imager plate (Fig. 6). A major band with an apparent molecular mass of 70–75 kDa is clearly evident. We interpret this band to be a ligand-receptor complex consisting of a monomer of [125I]BMP-9, 13 kDa in size, cross-linked to a >60-kDa receptor. The signal at the bottom represents monomeric [125I]BMP-9 that was not cross-linked. In addition, the radiolabeled ligand-receptor complex was not seen when the indicated concentrations of unlabeled rhBMP-9 were added. Finally, the formation of the radiolabeled complex is dependent upon the presence of BS3. The experiment was performed twice yielding identical results.

To demonstrate the specificity of BMP-9 binding to LEC and KC we attempted to compete for the [125I]BMP-9-binding site with other members of the TGF-β superfamily (Fig. 7). Using 2 ng/ml radioligand in an overnight incubation at 4 °C, we established maximum binding for both LEC (Fig. 7A) and KC (Fig. 7B). Competitor BMPs-2, -4, -6, -9, and -12 were used at 16 ng/ml (8-fold excess, filled bars) and 200 ng/ml (100-fold excess, open bars). Only BMP-9 was able to compete for radioligand-
binding sites on LECs and KCs. Comparing the amount of bound radioactivity in the presence of 16 ng/ml to that bound in the presence of 200 ng/ml unlabeled BMP-9, it was apparent that an 8-fold excess of BMP-9 established the nonspecific binding of [125I]BMP-9 to both of these cell types. Both concentrations of unlabeled BMP-9 reduced the amount of bound radioactivity by nearly 80%. When candidate competitor BMP-2, -4, -6, and -12 were provided at 100-fold excess of the BMP-9 radioligand, binding was reduced by approximately 10%. Preliminary results obtained using excess BMP-7 as a competitor have yielded similar results, suggesting that it is also unable to compete for the BMP-9 binding site on these cell types (see “Experimental Procedures” and data not shown).

We asked whether or not these cells could internalize the cell surface receptor-BMP-9 complex. Initially we determined that three acid wash cycles effectively remove more than 99% of the total BMP-9 bound (data not shown). In addition, we established that ligand internalization was temperature-dependent and that this process was minimal (approximately 10% bound radioactivity was internalized) when cells were incubated at 4 °C. LEC were incubated at 4 °C for 4 h in the presence of increasing concentrations of [125I]BMP-9. Acid wash-removable, internalized, and total bound [125I]BMP-9 were then determined (Table II)(see “Experimental Procedures”). A majority of the iodinated ligand remained on the cell surface when cells were maintained at 4 °C.

To examine whether or not BMP-9 receptors internalize this ligand under physiological conditions LECs were incubated at 37 °C in the presence of 300 pM [125I]BMP-9 for intervals up to 6 h. At the allotted times, acid wash-removable and cell asso-
Acid-washable radioactivity representing the \([^{125}\text{I}]\)BMP-9 bound to cell surface receptors initially increased as receptors were loaded with ligand and then decreased as the cell surface receptor population was reduced (Fig. 8A). Cell associated radioactivity representing internalized \([^{125}\text{I}]\)BMP-9 also increased initially, then superseded that which was on the cell surface and finally reached a plateau (Fig. 8A). It was apparent that LEC actively bound and internalized this ligand under these culturing conditions.

When this experiment was performed in the presence of an 8-fold excess of unlabeled ligand, the amount of cell surface and cell internalized radioactivity was reduced (Fig. 8A). This experiment demonstrated the specificity of the receptor-ligand
internalization event.

Also, this experiment was performed on primary cultures of KCs (Fig. 8B). In short, KC have the capacity to bind and internalize [125I]BMP-9 as well. As the incubation time increased, more radioactivity became refractory to the acid-wash procedure (Fig. 8B). The experiment was performed with KCs in the presence of 8-fold excess unlabeled BMP-9 to show that the internalization event was specific for this ligand (Fig. 8B).

The conditioned media from the internalization experiments discussed above were analyzed for metabolized fragments of [125I]BMP-9. Aliquots of serum-free conditioned media from LECs and KCs were resolved by SDS-PAGE. A single band of radioactive signal was detected that was indistinguishable from the signal produced by stock radioligand not exposed to cells. This observation indicated that digested fragments of [125I]BMP-9 were not a significant constituent in the conditioned media.

**DISCUSSION**

The initial study indicating that BMP-9 exhibits defined agonist responses in liver cells (17) suggested a myriad of questions to us concerning the origin and binding specificity of this TGF-β superfamily member. A survey of major organs revealed that the liver possesses a robust ability to synthesize this putative intercellular signaling molecule. This finding is interesting when considering the known expression patterns of other BMPs in adult mammals. For example, BMP-6 is expressed by muscle, lung, brain, skin, and gut (28). Numerous adult tissues have the capacity to express BMP-4 including spleen, lung, kidney, liver, and heart (6).

Other members of the TGF-β superfamily have interesting tissue-restricted expression patterns. In developing as well as adult mammals, GDF-8 is expressed exclusively by skeletal muscle (29). Removing this gene gave rise to an animal with...
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substantially increased skeletal muscle mass, indicating that this cytokine may regulate muscle growth (29). Of numerous adult organs surveyed for BMP-8A expression (30), only cells of both male and female reproductive systems transcribe this gene (31). In accordance with its organ-specific expression pattern, BMP-8A was found to be important in the maintenance of spermatogenesis in males (31).

Our results immediately suggest a liver-specific function for BMP-9. The fact that liver non-parenchymal cells reserve the capacity to synthesize and to bind this cytokine implies that BMP-9 signals mediate reticulendothelial system function within the liver. The cells comprising this system represent three functionally unique cell populations representing a multifaceted support system to the liver parenchyma. Our results suggest that there is an ongoing, basal level of transcription and translation of BMP-9 by the sinusoidal cell populations.

In accordance with the hypothesis that BMPs operate via autocrine and/or paracrine signaling mechanisms, we found a BMP-9 receptor in primary cultures of KC and LEC. This receptors affinity for BMP-9 is in the range previously reported for other members of the TGF-β superfamily (32). While there is abundant information available depicting BMP binding and cross-linking to transformed cells transfected with cloned receptors (13, 14, 33–35), there is relatively little published information explicitly defining BMP binding affinities or receptor populations in primary cell cultures. Primary human monocytes respond to BMP-2B receptor binding with chemotactic activity utilizing as few as 750 receptors per cell (15). BMP-4 has been shown to specifically bind primary bovine chondrocytes (Bmax 6000 rec/cell) and up-regulate extracellular matrix protein synthesis in these cells (16). A rather extensive survey of cell lines capable of binding BMP-2 shows that this BMP has picomolar affinity for receptors on several cell types (32).

The BMP-9 receptor binding activity and cell surface population appears to be of sufficient affinity and abundance to suggest physiological relevance. When analyzing BMP-9 binding to two different cell populations, similar binding parameters were found. KCs have an apparent Kd for BMP-9 that is only slightly less than that measured for LEC. KCs have fewer receptors/cell than LECs, e.g. the Bmax for KC appears to be approximately one-third of that found for LECs. In order to obtain the best fit of our data in Scatchard format, we found that it was necessary to invoke a negative cooperativity binding model in the Kupffer cells. The same model applied to liver endothelial cells did not significantly improve the quality of the fit. The biological implications of this observation will require more experimentation.

We were able to visually image this receptor in a covalently linked complex with the radioligand. When the molecular mass of the BMP-9 monomer is subtracted, we calculate the molecular mass of the receptor to be in the range of 60–63 kDa. This is slightly larger than the known BMP type I receptors, ALK-2, -3, and -6 which have been shown to be 53–58 kDa in size (33, 34). There is not a clear signal at the expected size for a type II receptor-ligand complex. Our data suggest that this band is likely to be a type I Ser/Thr kinase.

The specificity of the ligand-receptor interaction is a critical parameter that must be established in characterizing a relevant cytokine-receptor interaction. The mature TGF-β/BMP is a homodimer of 13–25-kDa subunits with 30–50% primary sequence homology (36). The fact that BMP-2, -4, -6, -7, -12, TGF-β1, and TGF-β2, were unable to displace [125I]BMP-9 indicates that this binding site is highly selective for BMP-9. Taking our binding specificity and cross-linking data together with the binding promiscuity and molecular weights of the known type I and II receptors, it is our contention that a novel BMP receptor has been identified in Kupffer cells.

Characterizing the cellular response to BMP-9 involved the investigation of potential cellular mechanisms involved in the processing the receptor bound cytokine. It is a common biological phenomenon for receptors of many different signaling pathways to be internalized upon binding their ligands. Among the possible consequences of receptor internalization is a change in the ability of the cell to respond to the ligand for that receptor. Some receptor/ligand systems, i.e. G protein-coupled and tyrosine kinase receptors, are subjected to this process as a means to turn off the signaling pathway or to remove the ligand from the receptor therefore rendering the receptor available for future signaling events (37, 38).

Transforming growth factor-β is internalized and metabolized by some cell types (20, 39). A single study has been published documenting BMP receptor internalization in skeletal muscle cells (40). Our results show that BMP-9 receptors are internalized in the presence of ligand, suggesting that the receptor can be down-regulated by its ligand. Indeed, beyond the 2-h time point, the amount of radioactivity inside both cell types exceeds that which remains on the cell surface. The available data suggest that BMP-receptor internalization in the presence of ligand may be a general mechanism used by BMP receptor-containing cells to process BMP signals.

It is known that TGF-β isotypes 1–3 as well as other BMPs including BMP-6 are expressed by mammalian liver (28, 41–43). TGF-β is considered to be a major factor in the progression of hepatic fibrotic and regenerative processes (44, 45). In addition it has been shown to modulate BMP-6 expression by hepatic stellate cells in culture (28). There is ample evidence that cytokines of this superfamily are present in the adult liver and that TGF-β itself is an integral part of disease processes in this organ. Further characterization of BMP-9 signaling in LECs and KCs downstream of receptor binding will enable a more thorough understanding of the physiological utility of this cytokine family in the context of liver function/dysfunction.

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