Modulation of $\alpha_1\beta_1$ integrin mediated adhesion of hepatocytes to collagen IV and laminin by divalent cations

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Cell matrix interactions play a critical role in hepatic development and regeneration after acute injury. These interactions are mediated by transmembrane receptors belonging mainly to the integrin family. We have tried to assess the role of divalent cations in mediating attachment of hepatocytes to matrix proteins like collagen IV (Col IV) and laminin (Ln). The three cations examined viz. Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ showed attachment promoting activity. Since $\alpha_1\beta_1$ integrin is a common receptor for col IV and LN in liver, the effect of cations in its binding to these matrix proteins was studied.

Although cations in general enhanced the binding, different cations exhibited differential effect in promoting the binding for different ligands. Mg$^{2+}$ ions were more effective in promoting the binding of $\alpha_1\beta_1$ integrin to col IV but Ca$^{2+}$ proved to be more effective one for Ln. Kinetic analysis of binding in dot blot assays using different concentrations of cations showed that while Mg$^{2+}$ was active at low concentrations Ca$^{2+}$ and Mn$^{2+}$ promoted the binding more at higher concentrations. Absence of competitive effect in binding studies showed that they bind at different sites on the receptor. Differential effects of cations in promoting the binding of $\alpha_1\beta_1$ integrin to col IV and Ln suggest that changes in level of diffusible cations can modulate affinity of the common receptor $\alpha_1\beta_1$ integrin to its ligands and can influence adhesion of hepatic cells to different matrix proteins during hepatic development and regeneration.

Integrins are a family of transmembrane heterodimeric cell surface receptors formed by the combination of $\alpha$ and $\beta$ chains which function to mediate the binding of cells to extracellular matrix (ECM) as well as other cells.$^{1-5}$ There are a number of different $\alpha$ and $\beta$ subunit combinations. Although they have similar target sequences, each receptor has a mutually exclusive specificity in ligand binding.$^6$ But, one integrin may show specificity to more than one ligand and similarly, different integrins may bind to a single ligand. For example, $\alpha_1\beta_1$ integrin binds to both collagen IV (Col IV) and laminin (Ln)$^{7,8}$, whereas $\alpha_4\beta_1$ and $\alpha_5\beta_1$ bind to fibronectin (Fn)$^9$ and Ln binds to $\alpha_2\beta_1$ and $\alpha_5\beta_1$.10

Divalent cations are required for the recognition of most ligands by integrins.$^{3,11}$ This requirement depends on the direct binding of cations to the integrin subunits and it is generally believed that the divalent cation binding and ligand binding sites are in close proximity to each other on integrins.$^{12-15}$ The presence of putative divalent cation binding domains on the $\alpha$ subunits of integrin and the critical role of extracellular divalent cations in integrin-ligand interactions suggest that structural alterations necessary for ligand recognition may occur as a result of divalent cation binding. The divalent cation, Mn$^{2+}$ augments ligand recognition for many integrins. But, on the whole, different integrins show different cation specificities. Elices et al. have shown that Ca$^{2+}$ inhibits the spreading of $\alpha_5\beta_3$ on Fn whereas Mn$^{2+}$ stimulates it.$^6$ Ca$^{2+}$ was found to have an inhibitory effect upon the functions of leukocyte function associated antigen (LFA-1)$^{17}$, and also on the Mg$^{2+}$ dependent adhesion of $\alpha_5\beta_1$inserted liposomes on different types of collagen.$^18$ But, both Ca$^{2+}$ and Mg$^{2+}$ increased the binding of $\alpha_5\beta_1$ to its ligands.$^{19}$

Integrin receptors are present in liver and changes in the distribution of adhesion receptors and their ligands occur during liver development and regeneration.$^{20}$ $\alpha_1\beta_1$ integrin has been isolated from adult liver and has been shown to bind to Col I, Col IV and Ln.$^{7,21}$ The possibility of diffusible cations modulating the interaction of the common receptor with Col IV and Ln was examined by studying the attachment of hepatocytes to different matrix proteins and by studying the in vitro binding of $\alpha_1\beta_1$ integrin to different ligands in presence of different cations and the results are reported here.
Materials and Methods

Materials

Plastic tissue culture dishes were purchased from Nunc (Denmark). Eagle's minimum essential medium and type IV Collagenase for liver perfusion were from Sigma Chem Co (St. Louis, MO). Col IV and Laminin from EHS tumor were a gift from C. Hughes, NIMR, London. [125I]-NaI was obtained form BARC, Bombay.

Methods

Hepatocytes were isolated from the liver of rats (Sprague-Dawley strain) by collagenase perfusion.

Attachment assay

Hepatocytes were washed with EDTA (10 mM), suspended in Tris (20 mM, pH 7.4)/135 mM NaCl, 5 mM KCl, 2 mM glutamine and 8 mM glucose in the presence and absence of CaCl2, MgCl2/MnCl2 and were seeded on culture plates, passively coated by laying Col IV (50 µg/ml) or Ln (50 µg/ml) and maintained at 37°C (5% CO2) in a Forma CO2 incubator. After the required time, medium with unattached cells and the attached cells were collected and the percentage attachment was measured as described by measuring the protein content.

Integrin was isolated by affinity chromatography over collagen sepharose, prepared by coupling Col I to sepharose 4B (ref. 26). The tissue (liver and placenta) was homogenised in hypotonic buffer (1 mM NaHCO3, 0.5 mM CaCl2, 1 mM PMSF) repeatedly and the plasma membrane isolated. It was then extracted with detergent buffer (0.025 M Tris/HCl pH 7.4 containing 0.15 M NaCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM PMSF and 1 mM each of CaCl2, MgCl2 and MnCl2) for 12 hr. at 4°C. The supernatant after pre-chromatography over sepharose 4B was subjected to affinity chromatography over Col I-sepharose. Bound protein was eluted using 20 mM EDTA in 25 mM Tris-HCl, pH 7.6. The protein rich fractions were pooled, dialysed against 10 mM Tris-HCl, pH 7.6 and lyophilised.

The α1β1 integrin isolated from tissues was labelled with [125I]-NaI using chloramine T (ref. 28). The radio-iodinated protein was dialysed and subjected to gel filtration over Sephadex G-25. Percentage organification, calculated by precipitating the protein using phosphotungstic acid was over 80%.

Dot-Blot assay

Nitrocellulose discs were coated with the respective ligand, blocked by incubating with 1% albumin for 30 min and washed twice in PBS. Discs were then incubated in the presence of iodinated protein. The binding was calculated by measuring the radioactivity of the supernatant and membrane separately in a LKB gamma counter.

Results

Effect of different cations on the attachment of hepatocytes to Col IV and Ln

The interaction of hepatocytes with matrix protein was studied by measuring the attachment of hepatocytes to culture plates passively coated with Col IV and Ln. The effect of cations on the attachment of hepatocytes to Col IV and LN was studied using hepatocytes pretreated with cation chelating agent, EDTA in medium deficient in cations. Addition of CaCl2, MgCl2 and MnCl2 caused an increase in the attachment of hepatocytes to Col IV and Ln (Fig 1). At 0.25 mM concentration of cations, maximum attachment promoting activity to Col IV was shown by Mg2+ while at this concentration of cations, maximum attachment of hepatocytes to Ln was produced by Ca2+ ions indicating a difference in the effect of cations in promoting attachment of hepatocytes to different matrix proteins. This was examined in detail by varying the concentration of different cations (Fig. 2 and Fig. 3). With increase in the concentration of Mg2+, there was an increase in the attachment of hepatocytes to Col IV and Ln substrata. Ca2+ and Mg2+ showed significantly more attachment promoting activity than Mn2+ to Col IV and Ln. The
effect of Ca\(^{2+}\) in promoting attachment was different from Mg\(^{2+}\) ions. At low concentrations (upto 1 mM) Mg\(^{2+}\) showed more attachment promoting activity to Col IV than Ca\(^{2+}\) while at higher concentrations Ca\(^{2+}\) was more effective in promoting binding to Col IV. At low concentration of Mg\(^{2+}\), the hepatocytes adhered more to Col IV while at higher concentrations the hepatocyte attachment to Ln was more, indicating a difference in the effect of divalent cations in promoting attachment to Col IV and Ln.

In the absence of cations, there was a significant attachment of cells to Col IV and Ln coated coverslips. The role of cell surface \(\alpha_1\beta_1\) integrin in mediating the attachment to Col IV and Ln was examined by pretreating cells with antibodies against \(\beta_1\) subunit. Antisera against \(\beta_1\) subunit, significantly reduced the effect of Ca\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) in promoting attachment to Col IV and Ln (Fig. 4). But the antiserum did not affect the attachment of cells to Col IV and Ln in a cation free medium, probably indicating that the effect of cations in promoting attachment is mediated through integrins.

**Effect of cations on the binding of \(\alpha_1\beta_1\) integrin to Col IV and Ln**

\(\alpha_1\beta_1\) integrin was isolated from liver and placenta and was radioiodinated and studied the effect of cations in mediating its binding to different ligands in dot blot assays. There was an increase in the amount of integrin bound with increase in the concentration of ligands and attained a maximum at 50 \(\mu\)g/ml. Binding also increased with increase in the amount of integrin. Maximum binding was observed in 1 hr.

Addition of cations, in general, increased the binding of \(\alpha_1\beta_1\) integrin to Col IV and Ln. In the case of Col IV, Mg\(^{2+}\) showed maximum activity at 0.5 mM concentration which decreased as concentration was increased (Fig. 5); but the Ca\(^{2+}\) and Mn\(^{2+}\) ions increased the binding with increasing concentration and at 1mM concentration the maximum activity was attained. But, when Ln was used as ligand a different pattern was followed by the cations in promoting the binding (Fig. 6). At both high and low concentrations the binding promoting effect was in the order Mn\(^{2+}\) > Mg\(^{2+}\) > Ca\(^{2+}\). The biphasic pattern shown by Mg\(^{2+}\) in the binding to Col IV was not found in the case of Ln.

In order to examine the tissue specificity, the binding of radioiodinated placental \(\alpha_1\beta_1\) integrin to Col IV and Ln was also studied. Binding to Col IV...
coated nitrocellulose discs was found to be considerably increased in the presence of all the three divalent cations, viz. Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$. For Col IV, at low concentrations of various cations, Mg$^{2+}$ was found to cause the maximum effect in promoting the binding of $\alpha_2\beta_1$ integrin. The relative activity of the cations in promoting binding of $\alpha_2\beta_1$ integrin to Col IV was in the order Mg$^{2+}$ > Mn$^{2+}$ > Ca$^{2+}$ at these low concentrations. But as concentration was increased the activity of both Ca$^{2+}$ and Mn$^{2+}$ increased, and at higher concentrations, the binding promoting activity was in the order Mn$^{2+}$ > Ca$^{2+}$ > Mg$^{2+}$. The activity of all the three cations reached saturation at 1 mM concentration (Fig. 7). In the case of Ln, the binding of $[^{125}\text{I}]\alpha_2\beta_1$ integrin was increased in the presence of all the three cations, but the pattern was different from that of Col IV. At both high and low concentration of cations, the binding promoting activity of the three cations was in the order Ca$^{2+}$ > Mn$^{2+}$ > Mg$^{2+}$ (Fig. 8). The binding to Ln was found to increase with increase in the concentration of cations similar to the hepatic $\alpha_2\beta_1$ integrin.

**Binding of $\alpha_2\beta_1$ integrin to Col IV and Ln in the combined presence of different cations**

In order to study whether different cations promote binding of ligands to integrin by interaction with same or different sites, the binding was examined using different combinations of cations. When the binding was studied with the same concentration of Mg$^{2+}$ as in the above experiment but in the presence of 0.5 mM Ca$^{2+}$, the biphasic effect of Mg$^{2+}$ was not observed (Fig. 9). At 0.5 mM concentration, the activity was high but on further increase in concentration, the activity did not decrease. As the concentration of
Mg\textsuperscript{2+} was increased, the binding of \(\alpha_\text{IIb}\beta_\text{3}\) integrin to Col IV also increased in the presence of Ca\textsuperscript{2+}. At 1 mM concentration the binding was maximum. When Ln was the ligand, the binding increased with increase in the concentration of Mg\textsuperscript{2+} attaining a maximum at 1 mM [Mg\textsuperscript{2+}]. The binding promoting activity was higher than in the absence of Ca\textsuperscript{2+}. Conversely, when different concentrations of Ca\textsuperscript{2+} were used in the presence of 0.5 mM, Mg\textsuperscript{2+} the binding was very high at low concentrations unlike in the case of Ca\textsuperscript{2+} alone (figure not shown). With further increase in concentration there was a slight increase in the binding but it reached a maximum at 1 mM concentration of Ca\textsuperscript{2+}. For Ln, the binding increased with increase in concentration and reached saturation at 1.5 mM. The total binding was more than that caused by Ca\textsuperscript{2+} alone.

For Col IV substratum, when [Mg\textsuperscript{2+}] was altered in the presence of 0.5 mM MnCl\textsubscript{2}, the binding was similar to the one observed for Mg\textsuperscript{2+} alone (figure not shown). There was an initial increase at very low concentration followed by a decrease in binding at concentration above 0.5 mM. Mn\textsuperscript{2+} did not cause any alteration in the binding promoting activity of Mg\textsuperscript{2+}. The increased binding at low concentrations of MgCl\textsubscript{2} was not retained at high concentrations in the presence of Mn\textsuperscript{2+}. On the other hand, when Mn\textsuperscript{2+} concentration was altered, by keeping a constant (0.5 mM) concentration of MgCl\textsubscript{2}, (Fig. 10) there was an initial increase followed by a steep decline and then an increase in binding. For Ln substratum, the presence of Mn\textsuperscript{2+} caused greater binding than in the presence of Mg\textsuperscript{2+} alone. Increased binding was observed by increasing concentrations of Mn\textsuperscript{2+} in the presence of Mg\textsuperscript{2+} alone. But for both ligands, there was an increase in the total binding compared with the individual cations taken alone.

**Discussion**

Kinetic analysis of hepatocyte attachment to matrix proteins like Col IV and Ln in the presence and absence of divalent cations showed that they can modulate hepatocyte-matrix interactions. Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and Mn\textsuperscript{2+} increased the percentage attachment of hepatocytes to both Col IV and Ln substrata. But there was considerable attachment in the absence of cations as well. This indicates the existence of cation independent attachment of cells and the presence of non-integrin receptors on the cell surface which mediate cation independent interaction with matrix protein\textsuperscript{29-34}. The blocking effect of \(\beta_\text{3}\) integrin antiserum on the cation dependent attachment of hepatocytes to Col IV and Ln suggests that the cation dependent cell adhesion is mediated mainly through integrin receptors.

The binding properties of both liver and placental \(\alpha_\text{IIb}\beta_\text{3}\) integrin to Col IV and Ln in solid phase assay was found to be influenced positively by the cations. But the effects of cations in promoting binding of \(\alpha_\text{IIb}\beta_\text{3}\) integrin to different ligands were different for different cations. The binding of \(\alpha_\text{IIb}\beta_\text{3}\) integrin to Col IV was increased at low concentrations of Mg\textsuperscript{2+} but a relatively higher concentration of Ca\textsuperscript{2+} was required to produce such higher binding. But for Ln, at high concentrations of Mg\textsuperscript{2+}, the binding increased unlike...
in the case of Col IV. Thus, it appears that at low concentrations of Mg$^{2+}$ more of $\alpha_3\beta_1$ integrin binds to Col IV while an increase in Mg$^{2+}$ concentration reduces/weakened binding to Col IV and increases binding to Ln. Similarly, at higher concentrations of Ca$^{2+}$, binding of $\alpha_3\beta_1$ integrin to Ln is less as compared to that of Col IV, so that an increase in Ca$^{2+}$ concentration can favour the binding of $\alpha_3\beta_1$ integrin to Col IV than to Ln. A decrease in the concentration of a particular ion and increase in that of another ion can thus change the binding of $\alpha_3\beta_1$ integrin to a particular ligand. For example, when high concentration of Ca$^{2+}$ is replaced by high concentration of Mg$^{2+}$, binding of $\alpha_3\beta_1$ integrin to Col IV is weakened. An obvious physiological implication is that the change in the concentration of certain diffusible cations can cause change in the adhesive properties of cells to different ligands, which may have important implications in cell migration.

Earlier studies with $\alpha_3\beta_1$ integrin have also highlighted these differential effects of cations in their binding properties to different ligands like Col IV, Ln and Fn$^{16}$. Our results show that Ca$^{2+}$ supports ligand binding which has been proved to be true for many other integrins, especially of the $\beta_1$ family$^{35}$. But in some cases, it is found to have an inhibitory effect. Strong inhibitory effects with Ca$^{2+}$ have been reported in $\alpha_5\beta_1$ binding to Col$^{36}$ and $\alpha_5\beta_1$ binding to Ln$^{37}$. Ca$^{2+}$ did not support the binding of $\alpha_5\beta_1$ to Fn and also inhibited Mn$^{2+}$ binding$^{38}$. Although the binding characteristics of both hepatic and placental $\alpha_3\beta_1$ integrin to Col IV and Ln are essentially similar, there are some minor differences which may be attributed to the tissue-specific properties of integrins. This type of tissue-specific binding was also observed for $\alpha_5\beta_1$. The integrin $\alpha_3\beta_1$, from HT1080 cells bind to immobilized Col I and II unlike that from melanoma cells$^{30-32}$. Studies by Bazzoni et al.$^{41}$ have shown that the cations differentially induce conformational changes in $\beta_1$ integrin favourable for ligand binding. Ca$^{2+}$ inhibits these conformational changes while Mn$^{2+}$ enhances it. This is comparable to our results in which $\alpha_3$ integrin binds to different ligands depending on the presence or absence of the specific cations. Thus, at lower concentrations of cations, Ca$^{2+}$ promotes the binding of $\alpha_3\beta_1$ integrin to Ln, whereas its binding to Col IV is promoted by Mg$^{2+}$ ion.

The competitive binding studies with different cations suggest that the cations are binding at apparently different sites. Binding to Col IV was significantly high at very low concentrations of Mg$^{2+}$, suggesting that the binding site of Mg$^{2+}$ may be of high affinity. On the other hand, Ca$^{2+}$ and Mn$^{2+}$ binding sites appear to be of low affinity since the binding was increased only at higher concentrations of these cations. But for Ln, the Mg$^{2+}$ binding site also does not appear to be of high affinity. The effect of Ca$^{2+}$ in promoting binding of $\alpha_3\beta_1$ integrin to Col IV was not affected by Mg$^{2+}$. Similarly, the increased binding at low concentrations of Mg$^{2+}$ was unaffected by the presence of Ca$^{2+}$ or Mn$^{2+}$. For Ln also, there is no competitive effect by any of these three cations. Thus, all these cations appear to have distinct independent binding sites on $\alpha_3\beta_1$ integrin and affect differently the binding to both Col IV and Ln ligands.

It has been suggested that in the binding of $\alpha_3\beta_1$ to osteopontin, Ca$^{2+}$ only decreased the association rate of Mn$^{2+}$ supported ligand binding and that Mn$^{2+}$ and Ca$^{2+}$ bind to different sites on the integrin$^{32}$. Mould et al.$^{40}$ suggest that there are cation binding sites on $\alpha_3\beta_1$ integrin that selectively bind only one type of divalent cation.

ECM appears to play a critical role during hepatogenesis and regeneration after partial hepatectomy.$^{44}$ It contributes to maintenance of characteristic tissue architecture and the differentiated state of various cell types. In rat by day 13.5 of gestation, hepatoblasts organise into cell plate and the microvasculature is lined by discontinuous fenestrated endothelial cells. Although from this time on, subsinusoidal space contains occasional discontinuous deposits of Col IV and Ln, no basement membrane is found. During early gestational phase, Col IV is less prevalent than Ln. After 18 days of gestation, ratio of Col IV to Ln increases and on day 21, adult liver architecture is attained, where Disse's space appears having occasional discontinuous deposits of Col IV. Similarly, during hepatic regeneration after partial hepatectomy, changes in distribution of basement membrane components occur and suggest changes in the cell matrix contacts particularly with Col IV and Ln. The proliferating cells have to establish contact with appropriate components of the matrix. In addition to changes in the matrix components, temporal and spatial changes occurring in integrin receptors for matrix proteins can cause alteration in cell matrix contacts. Apart from changes in the cell surface receptors, alterations in the affinity of cell surface integrin receptor to its ligands can result in weakening of cell matrix contacts or formation and stabilisation of new cell matrix contacts. Results
presented here indicate that cations differentially modulate the affinity of $\alpha_\beta_1$ integrin to its ligands viz. Col IV and Ln, whose level and distribution changes during hepatic development. The modulation of the affinity of the integrin receptor by diffusible cations may also contribute to cell migration during development and tissue morphogenesis where cell contacts with matrix components such as Col IV and Ln change alternatively. It, therefore, appears that the diffusible cations, by modulating the affinity of the common $\alpha_\beta_1$ integrin to its different ligands can influence adhesion of hepatic cells to different matrix proteins during hepatic development and regeneration.

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