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A proposed mechanism for material-induced heterotopic ossification

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Repairing large bone defects caused by severe trauma or tumor resection remains one of the major challenges in orthopedics and maxillofacial surgery. A promising therapeutic approach is the use of osteoinductive materials, i.e. materials able to drive mesenchymal stem cells into the osteogenic lineage. Even though the mechanism of this so-called intrinsic osteoinduction or material-induced heterotopic ossification has been studied for decades, the process behind it remains unknown, thus preventing any design of highly potent osteoinductive materials. We propose and demonstrate for the first time that intrinsic osteoinduction is the result of calcium and/or phosphate depletion, thus explaining why not only the material (surface) composition but also the material volume and architecture (e.g. porosity, pore size) play a decisive role in this process.

Introduction

The skeleton provides mobility, support for the organs, and has a very important esthetic function. Therefore, a loss of skeletal integrity can have dramatic consequences, such as a reduction in life expectancy [1,2], and a poor well-being following disfiguration [3]. Various therapeutic approaches have been proposed for the treatment of large bone defects, but all present important drawbacks. Indeed, surgical techniques such as the Ilizarov technique [4,5], the Masquelet technique [6], or vascularized flaps [7] are challenging for the patient and the surgeon, require several surgical steps spread over months, and may lead to complications [8]. Bone morphogenetic proteins (BMPs) have generally been associated with positive clinical outcomes [9–11]. However, several limitations have also been reported including additional material costs and excessive BMP dose leading to potential inflammation/edema [12]. In fact, the European Commission has withdrawn its approval of "InductOs®" (BMP2) product in 2015 [13]. Lastly, tissue engineering strategies [14] including bone marrow extraction [15], platelet-rich plasma [16], and other regenerative biomaterials have all displayed limited regenerative potential. Therefore, the reconstruction of large bone defects remains a prominent clinical challenge requiring alternative approaches.

In 1969, Winter and Simpson described the formation of bone in a polyhydroxyethylmethacrylate sponge implanted subcutaneously in pigs after 6 months [17]. Since then, various metals [18–21], composites, [22] and ceramics have demonstrated their ability to trigger bone formation in heterotopic (= ectopic) sites, what researchers sometimes refer to as "intrinsic osteoinduction" [23]. This should not be confused with "osteoinduction" which refers to the (material-free) "induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells" [24]. In 2010, Yuan et al. [25] showed equivalent potential for new bone formation in an orthotopic sheep model at 12 weeks of a BMP2 product, autograft, and a synthetic β-tricalcium phosphate (β-TCP; β-$Ca_3(PO_4)_2$). In 2017, a prospective study of lumbar interbody fusion rates in humans reported almost equivalence between a 95% β-TCP-5% hydroxyapatite product and a BMP2 product [26]. Unfortunately, "intrinsic osteoinduction" [23] may happen months or years after implantation and is considered unpredictable in various animal models. In addition, the mechanism of intrinsic osteoinduction remains unknown [21,23,27–42],

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which prevents any rational design of more sophisticated and potent osteoinductive biomaterials than the currently reported osteoinductive biomaterials.

The mechanism of intrinsic osteoinduction is often related to the release of calcium and phosphate ions. Ripamonti et al. [43] speculated for example that calcium ion release plays a key role for angiogenesis, and stem cell differentiation. For Habibovic et al. [44], the release of calcium and phosphate ions must be followed by the precipitation of a "biological apatite layer", which can then bind or adsorb osteogenic proteins [28]. The aim of this study is to demonstrate that it is in fact not the local accumulation/release but the local consumption/depletion of calcium and phosphate ions through apatite formation that is at the origin of intrinsic osteoinduction. With this very simple conceptual change, which is supported by theoretical and experimental evidence, it is possible to explain a number of currently unexplained findings, such as why materials devoid of calcium phosphates like metals and polymers [17–21] are sometimes osteoinductive, why intrinsic osteoinduction takes weeks to months to occur, and why ectopic bone formation happens first in the core of implanted materials [18,29,44–47]. It could also provide an explanation for (material-free) heterotopic ossifications.

Prior to going into the details of the newly proposed mechanism, the most important observations that have been made in the past 50 years on intrinsic osteoinduction are recapitulated. The mechanism that is generally considered to explain intrinsic osteoinduction is then critically assessed. Lastly, the new proposed mechanism is explained and discussed with an attempt being made to relate material-induced ("intrinsic osteoinduction") and material-free heterotopic ossification.

Intrinsic osteoinduction relies on physical, chemical, and biological factors

The observations made over the past 50 years in the field of intrinsic osteoinduction underline the importance of physical, chemical, and biological factors for this currently unexplained phenomenon:

(a) The formation of a biomimetic apatite layer on the material is a pre-requisite [17,18,20,22,23,47], but not a determinant for intrinsic osteoinduction [48,49].
(b) Intrinsic osteoinduction happens first on the surface of pores present in the core of a material, and then spread toward the periphery [18,29,44–47]. This contrasts with osteoconduction, which starts first at the periphery and then spreads into the material (Fig. 1) [34].
(c) Intrinsic osteoinduction is more often seen in large animals than in small animals [33,34,50–52].
(d) The scaffold architecture plays a very important role for intrinsic osteoinduction. Bone is generally found in concavities rather than convexities, and an increase of microporosity positively affects osteoinduction [25,48,53–57].
(e) Intrinsic osteoinduction does not depend on the chemical composition because it has been observed in polymers [17], metals [19–21], calcium phosphate-polymer composites [22], and calcium phosphates. However, calcium phosphates are particularly prone to induce bone formation [23,28].
(f) Ingrowth of blood vessels into the material is a necessary [56,57] but not sufficient condition for intrinsic osteoinduction.
(g) Intrinsic osteoinduction is a very slow process: bone formation may take a few weeks up to one year to occur [23,46,58]. This is in contrast with the very rapid (1–3 days) woven bone formation in bone defect healing [59].
(h) Even though both calcium and phosphate ions are considered to play a key role in intrinsic osteoinduction [28,33,35,44,47,48], a large number of studies point out to the importance of Ca ions and the Ca sensing receptor [25,27,31,40,54,57,60–62].
(i) Cartilage formation has been observed and suggested to occur during intrinsic osteoinduction [53,63], but it is generally admitted that intrinsic osteoinduction provokes an intramembranous ossification [28,30,33,64] with the formation of woven bone [17,29,30,45,48,51,60,65] and then lamellar bone.
(j) Macrophages and osteoclasts [29,32,36,41–43,66–68] are considered to play an essential function in intrinsic osteoinduction.

The mechanism of intrinsic osteoinduction is unknown

Intrinsic osteoinduction is a complex process involving physical, chemical, and biological factors. This was already recognized by Yamasaki in 1990 [69]. In 1991, Ripamonti [45] underlined the importance of surfaces, and speculated that "circulating or locally produced growth and inducing factors, or both," adsorbed on the scaffold and then were released during "mesenchymal-tissue invasion" leading to "the differentiation of bone". This concept has only slightly evolved over the years. It is generally assumed that growth factors are included into the biomimetic apatite layer that forms prior to bone formation from a "continuous dissolution-precipitation" [28,70,71]. Differentiation of MSCs is then provoked by surface topography, or by the action of inflammatory cells (monocytes, macrophages, osteoclasts) resulting in the release of growth factors, calcium and phosphate ions [28]. Additionally, it has been speculated that Ca and phosphate ions can better accumulate in the material cores, in materials with higher surface area, and in concave versus convex pores [28,44]. Unfortunately, this mechanism does not explain a number of experimental findings, such as why materials devoid of calcium phosphates like metals and polymers [17–21] are sometimes osteoinductive, why intrinsic osteoinduction takes weeks to months to occur, and why ectopic bone formation happens first in the core of implanted materials [18,29,44–47]. Also, the involvement of endogenous growth factors put forward by Ripamonti [45] is questioned by the fact that ossification in intrinsic osteoinduction does not proceed endochondrally, as often observed with growth factors, but intramembraneously [28,30,64]. In fact, there is a general agreement that the mechanism of intrinsic osteoinduction is unknown or at best unclear [21,23,27–42].

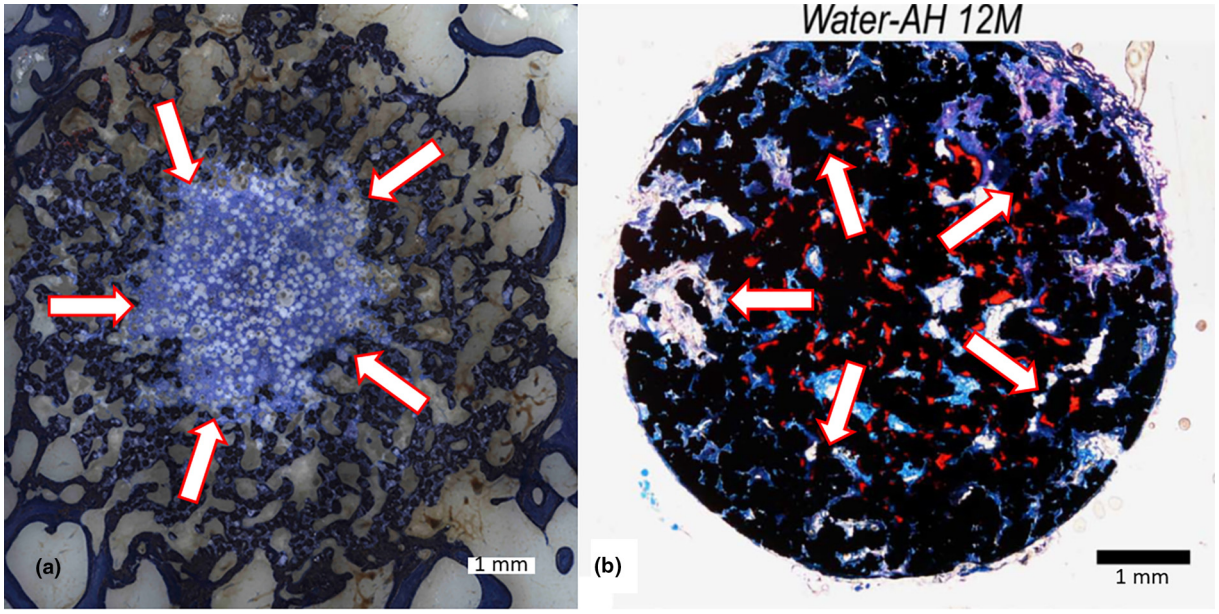

FIGURE 1

Illustration of the difference of bone formation (a) in an orthotopic site (bone = dark blue color; material: β-tricalcium phosphate) and (b) in a heterotopic site (bone = red color; material: Titanium) (Adapted from Takemoto et al. [18]). The arrows indicate the direction of bone formation: inward for osteoconduction; outward for osteoinduction.

No accumulation of calcium and phosphate ions within osteoinductive materials

One popular mechanism used to explain intrinsic osteoinduction is based on the assumption that there is at some point during implantation the release of calcium and phosphate ions, thus leading to supra-physiological calcium and phosphate concentrations. These supra-physiological concentrations are assumed to drive stem cells into the osteogenic lineage. The opposite seems to be much more likely. This statement is not only based on in vitro and in vivo data, but also by thermodynamic considerations. Indeed, calcium and phosphate concentrations decrease in cell culture media in contact with osteoinductive materials due to apatite precipitation [31,70–74]. In vivo, the formation of a biomimetic layer (observation "a" herein), which by definition consumes calcium and phosphate ions, is a prerequisite for intrinsic osteoinduction [17,20,22,23,47]. Thermodynamically, physiological fluids are supersaturated toward hydroxyapatite at pH 7.4 [75–77]. According to Bohner and Lemaître [75], the supersaturation of serum is in the range of $10^{1.4}$ (≈25) which means in a very crude approximation that 96% of all calcium and phosphate ions could precipitate to reach the chemical equilibrium between hydroxyapatite and serum. In healthy human beings, soft tissue mineralization does not occur due to the presence of nucleation and growth inhibitor such as proteins, citrate or Mg ions. However, as stated by Posner [78], this metastable state can be disturbed by a local increase of supersaturation (e.g. increase of pH or Ca concentration), by the neutralization of bone mineral inhibitors, or by providing substances which create nucleation sites or remove barriers to these sites (e.g. collagen matrix). The implantation of a bone substitute falls in the latter category because it may act as nucleation site for the precipitation of apatite crystals, thus triggering mineralization via the so-called heterogeneous precipitation. This is the "bioactivity" concept introduced by Kokubo [79]. A demonstration of this concept may be conceptualized by the observation that the in vivo weight of a sintered hydroxyapatite block continuously increases during implantation into soft tissue [80]. Interestingly, several studies show that an increase of in vitro bioactivity leads to an increase in intrinsic osteoinduction [18,20,21,40,57].

Osteoclastic resorption does not trigger intrinsic osteoinduction

Osteoclast-mediated resorption is mentioned to explain the release and thus accumulation of calcium and phosphate ions in the core of osteoinductive materials. However, a number of studies have reported the absence of resorption of scaffolds prior to bone formation [34,41,52,67,81,82]. Also, cell culture studies of MSCs have shown that differentiation of MSCs into the osteogenic lineage may occur in the absence of osteoclasts [40,62,70,83]. Additionally, there is contradictory evidence about the role of osteoclast stimulation/inhibition on osteoinduction [36,42,84]. Furthermore, woven bone, the first bone seen during intrinsic osteoinduction, is known to start forming before osteoclastic resorption [59,85]. Finally, β-TCP has a lower osteoinductive potential than hydroxyapatite (HA) and biphasic calcium phosphate (BCP; mixture of HA and β-TCP), even though it is more resorbable [28,48]. Similarly, α-tricalcium phosphate, which is more soluble than β-TCP and prone to form hydroxyapatite in vivo, is not osteoinductive [28,86]. To summarize, it appears likely that intrinsic osteoinduction is not triggered by osteoclasts. However, osteoclasts may still play an important role in the events leading to intrinsic osteoinduction, as discussed hereafter.

New mechanism and paradigm for intrinsic osteoinduction

Based on the above, we propose that intrinsic osteoinduction is not caused by an accumulation of but by a reduction in the local calcium and/or phosphate ion concentration. This is possible if the local consumption of calcium and phosphate ions due to the precipitation of carbonated apatite (dahlite) [87] is larger than the supply of these ions by diffusion and convection processes (blood supply). The statement about the local depletion in calcium and phosphate concentration is supported by direct evidence (calcium and phosphate concentration drop during cell culture tests [31,69–73]) and indirect evidence (the formation of a bioactive apatite layer, which by definition consume calcium and phosphates ions, is always seen prior to intrinsic osteoinduction [17,20,22,23,47]). We propose therefore the following paradigm for intrinsic osteoinduction:

A material is osteoinductive if:

(C1) it mineralizes *in vivo* (formation of a "bioactive" apatite layer on the material thus consuming calcium and phosphate ions) [17,20,22,23,47].
(C2) it is porous (porous scaffold or assembly of granules).
(C3) the pores are large enough to allow blood vessels ingrowth and cell transport into the core of the material [56]. The minimum pore interconnection size can be inferred to be well below 50 μm [56,88].
(C4) blood supply is insufficient to maintain physiological calcium and/or phosphate ion concentrations.

Assuming that there are roughly 4 times more hydrogen phosphate ions than dihydrogen phosphate ions at pH 7.4 [89], the chemical reaction consuming calcium and phosphate ions to produce carbonated apatite (= dahlite), the bone mineral [87], can be written:

$$8.8Ca^{2+} + 1.1H_2PO_{4^-} + 4.1HPO_{4^{2-}} + 0.7HCO_{3^-} + 1.3H_2O \rightarrow Ca_{8.8}(HPO_4)_{0.7}(PO_4)_{4.5}(CO_3)_{0.7}(OH)_{1.3} + 7.6H^+$$

Blair et al. [90] proposed a simpler equation: $6HPO_4^{2-} + 2H_2O + 10Ca^{2+} \leftrightarrow Ca_{10}(PO_4)_6(OH)_2 + 8H^+$.

The proposed mechanism is not markedly different from the concept of "biological apatite formation" proposed by Habibovic et al. [44] except that it excludes the concept of "dissolution" and "calcium release" prior to apatite precipitation. It does neither include, nor exclude the involvement of growth factors [91] or inflammation [23,29,49].

Explaining past observations

With this mechanism, it becomes possible to understand the observations "a" to "g" mentioned herein:

(a) The formation of a biomimetic apatite layer on the material is a pre-requisite [17,20,22,23,47], but not a determinant for intrinsic osteoinduction [48,49]. Indeed, not all bioactive materials fulfill condition C4 ("insufficiently blood supply"). This is in particular the case at the surface of bioactive materials.
(b) Intrinsic osteoinduction happens first inside the pores of a material, and then spreads to the periphery [18,21,45,47]. As explained above, condition C4 is more easily met in the core of porous implants (Fig. 2).
(c) Intrinsic osteoinduction is more often seen in large animals [33,34,50–52]. It is well known that small animals have a faster metabolism than large animals and therefore a higher blood supply per volume [92]. As such, it is more difficult for small animals to fulfill condition C4. This

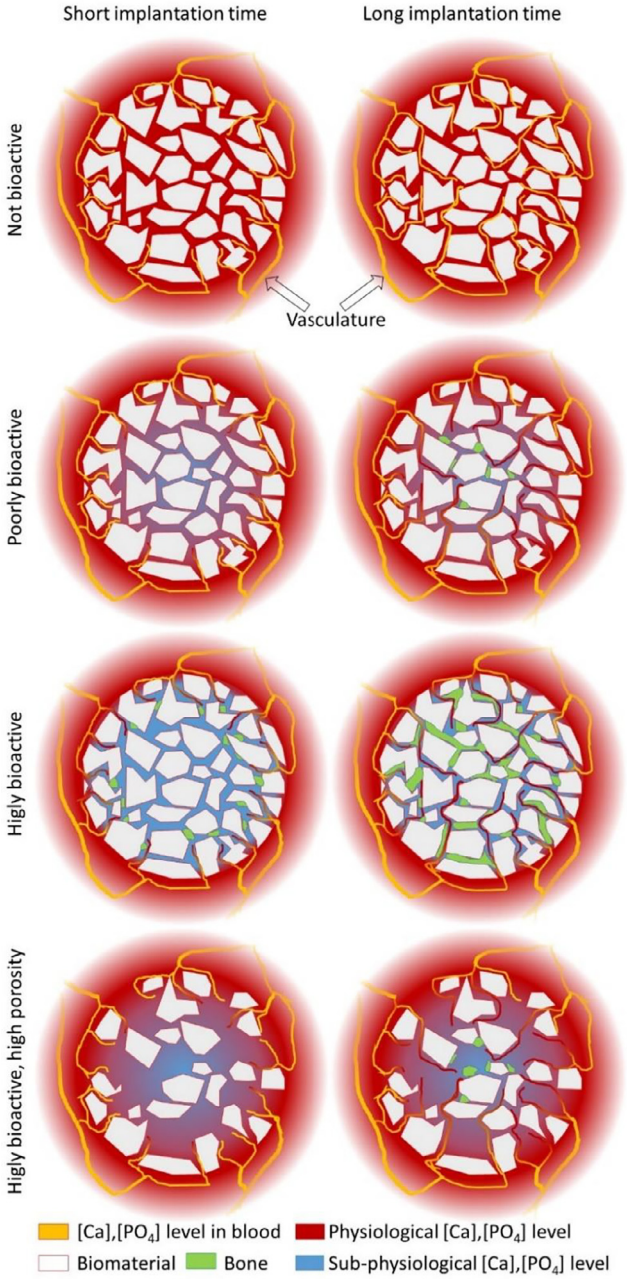

FIGURE 2

Schematic representation of blood vessel ingrowth and heterotopic bone formation in non-bioactive, poorly bioactive, and highly bioactive granular bone substitutes. The effect of a difference of porosity is also displayed. A higher osteoinduction is expected when the material bioactivity is increased and when the material porosity is reduced (provided blood vessel ingrowth is still possible). Total [Ca] in plasma/serum: 2.1–2.6 mM; Interstitial total [Ca]: ≈1.6 mM; [Ca] closed to hydroxyapatite: probably close to 0.1 mM (Table 1).

effect is reinforced when the implanted material volume is reduced in small animals.

(d) Condition C4 implies that the scaffold architecture must play a very important role for intrinsic osteoinduction. Indeed, condition C4 is more likely to be met in concavities (= closer to the material core) rather than in convexities, and when the material surface is enlarged, for example with an increase of microporosity [25,48,53–57] (more apatite formation per unit volume). An increase of macropore size or porosity is also detrimental to intrinsic osteoinduction (Fig. 2).

(e) All materials that trigger the formation of a biomimetic apatite layer can be osteoinductive, thus explaining why polymers [17], metals [19–21], calcium phosphate-polymer composites [22], and calcium phosphates have all been observed to possess intrinsic osteoinduction. Since apatite compounds like HA and BCP already contain hydroxyapatite crystals, they are particularly prone to fulfill condition C1 and hence trigger intrinsic osteoinduction [23,28].

(f) Ingrowth of blood vessels into the material is a necessary [56,57] (condition C3) but not sufficient condition for intrinsic osteoinduction because not all vascularized tissues are remote enough to fulfill condition C4.

(g) Intrinsic osteoinduction is a very slow process: bone formation may take a few weeks up to one year to occur [23,46,58]. This is related to the time it takes (i) for the biomimetic apatite layer to form and (ii) for blood vessels to grow into the scaffold and to bring the cells that will eventually trigger the osteoinductive response (Fig. 2). Apatite layer formation may easily take weeks to months to occur. For example, the ISO 23317:2014(E) standard testing the "in vitro evaluation for apatite-forming ability of implant materials" recommends to perform the test during 4 weeks. Even though the bioactive layer would form spontaneously, it is likely that intrinsic osteoinduction would only start after a few weeks because blood vessels ingrowth is in the order of a few hundred micrometers per day. For example, Nomi et al. [93] showed that blood vessels may take 1–2 weeks to penetrate a 3-mm-thick scaffold. Osteoblasts (245–662 μm/day) [94] and woven bone (<100 μm/day) [88,95] have similar motility/ingrowth rates.

Additional observations and remarks

We would like to point out a few interesting studies and explain how the observations made in these studies can be explained with the proposed mechanism. First, Fukuda et al. [21] observed that elongated pores promoted more new bone formation and closer to the surface when the diameter was reduced (Fig. 3). Since thinner cylindrical pores have a higher surface-to-volume ratio (proportional to d^{-1} where d is the pore diameter), more apatite should precipitate per volume, thus leading to higher ionic gradients, and using our mechanism, to earlier and closer to the pore orifice bone formation (Fig. 3). Considering also that bone formation spans over a period of 2–3 months [88], there is more bone in smaller pores and the maximum bone fraction is closer to the pore orifice. In another study, Wang et al. [56] observed that a decrease in granule size promoted osteoinduction (Fig. 3). In fact, they showed that smaller granule agglomerates had a smaller permeability, which is a good pre-requisite for the fulfillment of condition C4. They also showed that the smallest granules had no blood vessel ingrowth and accordingly no osteoinductive response (condition C3).

Shifting from physical to chemical factors, Tang et al. [28] stated that the propensity for calcium phosphates to trigger intrinsic osteoinduction is in the following order: BCP > β-TCP > HA ≫ α-TCP. α-TCP is soluble in physiological conditions, which means that it continuously releases calcium and phosphate ions. As such, the poor osteoinduction of α-TCP cannot be explained by the currently most accepted osteoinduction mechanism, i.e. the accumulation of Ca and/or phosphate ions, but can be easily understood under the newly proposed mechanism. Interestingly, α-TCP is "bioactive", i.e. it readily transform into calcium-deficient hydroxyapatite (CDHA). The reaction occurs at the external surface of α-TCP and is controlled by the diffusion of ions through the growing CDHA layer [96].

Contradictory results have been published on the intrinsic osteoinduction of β-TCP. It is sometimes considered to have limited or no osteoinduction [48,81,97], and in other cases be very osteoinductive [25]. This may be related to the inconsistent bioactivity of β-TCP, which is a pre-requisite for osteoinduction (see point "a" herein and condition C1). TEM studies of implanted β-TCP scaffolds did not reveal any formation of a bioactive (= apatite) layer prior to bone bonding [98,99]. However, it is known that β-TCP bioactivity can be triggered by autoclaving [100], and a number of heterotopic implantation studies revealing the intrinsic osteoinduction of β-TCP have used autoclaved β-TCP samples [37,81,101–105]. Therefore, we speculate that β-TCP osteoinduction varies according to its surface properties.

Ionic trigger for intrinsic osteoinduction

A number of cells have been considered to play an important role in intrinsic osteoinduction, such as stem cells [27,32,33,38,40,57,62,70,106], macrophages [29,32,66], osteoclasts [32,36,41–43,67,74], and pericytes [29,39,43,51,60]. The material-driven osteoinduction mechanism proposed herein cannot elucidate the biological mechanisms leading to intrinsic osteoinduction, but it underlines the potential role of calcium, phosphate, carbonate, hydronium ions, or a combination thereof (Eq. (1)).

Since a high local calcium concentration provoked by the action of osteoclasts is generally considered to trigger osteoinduction [25,27,31,40,54,57,60–62], two studies looked at the importance of osteoclasts and the Calcium-Sensing Receptor (CaSR) [107] on osteoinduction. They showed that blocking CaSR does indeed inhibit the osteoinductive response [62,84]. This suggests that CaSR and accordingly the calcium concentration is involved in intrinsic osteoinduction. Nevertheless, the Ca dose-dependency of this effect has not been elucidated yet.

Material-free heterotopic ossification

According to Baird and Kang [108], "heterotopic ossification (HO) is defined as the process by which trabecular bone forms

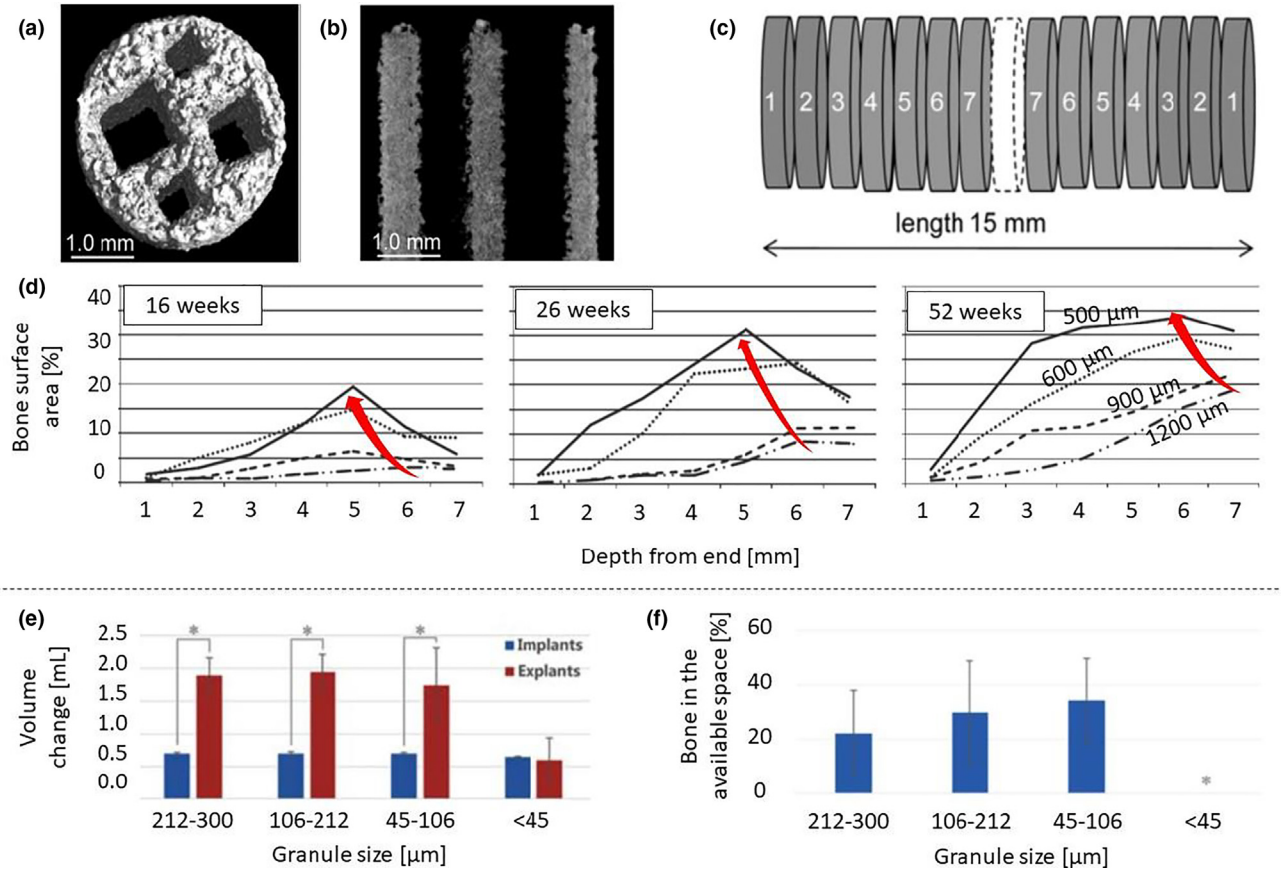

FIGURE 3

Experimental results of (A–D) Fukuda et al. [21] and (E, F) Wang et al. [56]. The Ti implants shown in (A, B) were implanted in the dorsal muscles of beagle dogs for 16, 26 and 52 weeks. The surface fraction of bone present in the implant pores was assessed for each of the four pore diameters (500, 600, 900 and 1200 µm) and for each of the 14 slices depicted in (C). The bone surface fraction increased with a decrease of pore size (see red arrows), an increase of the distance to the outside, and an increase of implantation time (D). The red arrows show that the position of the maximum of bone surface fraction moves toward the outer implant surface when the pore size is reduced. Wang et al. [56] implanted biphasic calcium phosphate granules of different sizes: <45 µm, 45–106 µm, 106–212 µm, and 212–300 µm. They observed an important swelling at the granules site (E) when bone was formed. Even though the difference was not significant, more bone was formed with a granule size reduction (F).

outside of the skeletal structure, occupying space in soft tissue where it does not normally exist". From that respect, it is similar to intrinsic osteoinduction, with the exception that it occurs in the absence of any implanted material. Prevalence may reach 50–90% [108–111] but remains mostly asymptomatic [110,112–114]. Three causes have been identified, namely neurological, genetic, and traumatic [110,115,116]. Interestingly, there are many similarities between intrinsic osteoinduction and trauma-induced heterotopic ossification: (i) the mechanism of heterotopic ossification is unclear [108,111,112,116]; (ii) it appears weeks to months after the traumatic or neurological event [110,115,116]; (iii) it starts by an edema/swelling [110,115,116] (seen also in granule-induced intrinsic osteoinduction [56] (Fig. 3). It is interesting to point out that Hung [117] (case report) associated edema formation to hypocalcaemia); (iv) precipitation of hydroxyapatite crystals occurs prior to ossification [108,116]; (v) size matters: the greater the trauma, the more likely it is that heterotopic ossification will develop [110,112]. It is therefore tempting to speculate that the same mechanism proposed here to explain intrinsic osteoinduction might also be involved in heterotopic ossification.

Conceptually, the first step of heterotopic ossification would be the precipitation of apatite crystals (Eq. (1)) in soft tissue (Fig. 4) [108]. This could be provoked by the release of matrix vesicles resulting from cell apoptosis [118] and/or the high calcium concentration prevailing at an injury site (= local supersaturation) [119]. These crystals would then grow and retrieve calcium and phosphate ions from the local environment. The initial mineralization is likely to occur within a few days after injury [111], but since apatite crystals are nano-sized (<100 nm), no signals would be observed by CT and MRI [111]. Provided blood supply is insufficient to keep physiological calcium and/or phosphate ion concentrations (condition C4), the low calcium and/or phosphate ion concentrations would then trigger a biological response eventually leading to heterotopic ossification. The difference in ossification pathways between intrinsic osteoinduction (intramembranous) and heterotopic ossification (mostly endochondral) [113,114,120,121] could be related to the presence/absence of a hard surface and/or differences in mechanical stability. Indeed, the mechanism of bone repair switches from endochondral to intramembranous ossification when fractures are treated by osteosynthesis rather than casts.

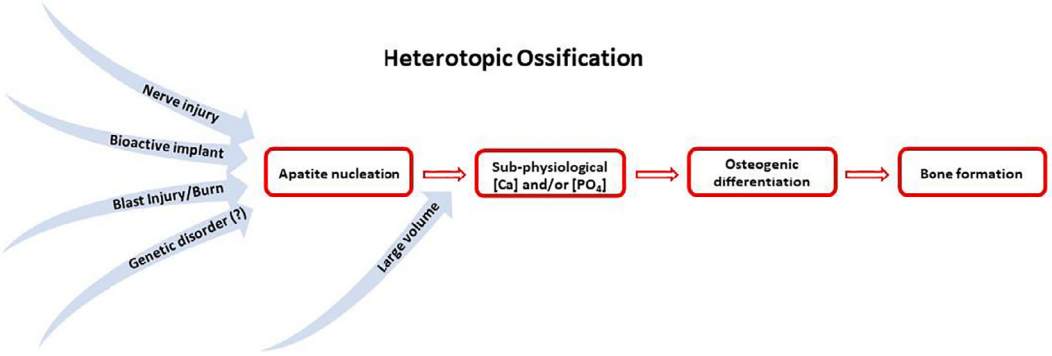

FIGURE 4

Schematic representation of the pathway leading to heterotopic ossification.

Biological mechanism involved in intrinsic osteoinduction

Experts in the field agree that chemical, physical, and biological aspects are involved in intrinsic osteoinduction. The physical (diffusion) and chemical (calcium phosphate precipitation) aspects have been discussed herein. In this last section, we would like to address the biological aspects.

During the process of intrinsic osteoinduction, stem cells are differentiated into osteoblasts. This simple statement hides many unanswered questions related to (i) the origin of the stem cells and (ii) the signal triggering the differentiation of stem cells into the osteoprogenitor lineage [28]. Regarding the first question, Song et al. [106] presented evidence that "stem cells can migrate from bone marrow through blood circulation to non-osseous bioceramic implant site to contribute to ectopic bone formation in a canine model". However, this does not exclude other stem cell origins, for example pericytic [29,39,43,51,60] or endothelial [51,60]. For Ripamonti [50,91], these stem cells would then be driven into the osteoprogenitor lineage by their interactions with growth factors present on the surface of the osteoinductive bone substitutes. The growth factors could be either adsorbed on the surface, secreted by local inflammatory cells, or integrated into the apatite (bioactive) layer formed on the material prior to the osteoinductive response [47]. A critical parameter would be the amount of proteins present on the surface [46,97]. However, this explanation is questioned in the literature [28] because the mechanism of bone formation is generally different in intrinsic osteoinduction (mostly intramembraneous) [28,30,33,64] compared to BMP-related osteoinduction (mostly endochondral). Calcium ions have also been discussed as chemotactic agent for bone marrow progenitor cells or pre-osteoblasts [62,122]. Currently, most efforts to understand stem cells differentiation in the context of intrinsic osteoinduction are dedicated to the role of the immune system, as described in more details hereafter.

Role of immune cells during intrinsic and heterotopic ossification

In order to fully appreciate the complexity of cell interactions during biomaterial integration, it is important to note that immune cells (namely monocytes and macrophages) are the first cell-type in contact with implanted biomaterials [123]. For many years, basic research focused on the ability of bone-forming osteoblasts to differentiate on various bone biomaterial surfaces. More recently however, it has been markedly determined that in fact macrophages and immune cells play a pivotal and substantial role demonstrating key functions during bone formation and remodeling [124]. Nevertheless, despite these essential findings convincingly showing the key role of macrophages during biomaterial integration [123], little information is available concerning their response to biomaterials with the majority of investigation primarily focused on their role during foreign body reactions. Today it is known that macrophages demonstrate extremely plastic phenotypes with the ability to differentiate toward classical pro-inflammatory M1 or tissue regenerative M2 macrophages. Macrophages play an important function during bone formation [125–127], play a key role during heterotopic ossification in various injury-related disorders [128–132], are highly implicated during calcification of arterial tissues [133,134], and associated with the heterotopic ossification of implanted biomaterials into soft tissues [68]. We therefore hypothesize that in each of the above-mentioned scenarios, changes in physiological calcium and/or phosphate levels within the local micro-environment may be a driving factor associated with bone-induction.

Role of macrophages during bone induction – studies from basic research

It is now understood that macrophages are the major effector cell during biomaterial integration where they are indispensable for osteogenesis. Various knockout models have demonstrated that a loss of macrophages around inductive BCPs entirely abolishes their ability to form ectopic bone formation, thus confirming the potent role of immune cell modulation during osteogenesis [66]. Ongoing research has further shown that macrophage deletion between days 0 and 3 following biomaterial implantation completely attenuates the ability for BCP grafts to induce ectopic bone formation, yet their later deletion (day 4 onward) has no effect on the graft's osteoinductive potential. It is also known that the fate of biomaterials is determined rapidly during the integration process. While the behavior of macrophages in calcium-rich and calcium-poor environments and their ability to polarize under various physiological conditions remains completely unstudied, future research aimed at better characterizing

TABLE 1

Calcium and phosphate concentrations in serum/plasma, interstitial fluids (between cells) and intracellularly. According to Bohner and Lemaître [68], the supersaturation of serum toward hydroxyapatite is in the range of $10^{1.4}$ (≈ 25) which means in a very crude approximation that 96% of all calcium and phosphate ions could precipitate to reach the chemical equilibrium between hydroxyapatite and serum (≈ 0.1 mM Ca and ≈ 0.04 mM phosphate). The values found in the literature for the concentrations in interstitial fluid and intracellular, in particular those of phosphate ions, are scarce and scattered over wide ranges. Interstitial fluids seem to have lower calcium and phosphate concentrations than serum/plasma.

	Serum/plasma		Interstitial fluids		Intracellular	
	Total	Ionized	Total	Ionized	Total	Ionized
Calcium	2.1–2.6 mM [156–160]	1.0–1.3 mM [156–158,160]	1.55 mM [158]	1.18 mM* [158]	Large**	0.0001 mM [161]
Phosphate	0.8–1.5 mM [89,156,158–160,162]	0.6–1.2 mM [89,158,160]	0.61 mM [158]		100 mM*** [162]	≈ 1 mM [160]

* This value is 7% lower than the ionized plasma level determined in the same study.
** The total content of calcium in cells may be large since e.g. osteoblasts contain Ca-rich matrix vesicles.
*** The reference states that this concentration is "100 times larger than extracellular".

their function around biomaterial's causing Ca/PO_4 depletion/accumulation may be key toward our understanding of osteoinductive events.

Prior to these discoveries however, complex studies from basic research have revealed the dynamic interactions between bone tissues and the immune system [125–127]. Over a decade has passed since it was revealed that macrophages (often referred to as OsteoMacs since they originate from osteal tissues) are the main effector cell responsible for dictating bone formation [126]. Although initial bone fracture healing experiments have been characterized by infiltration of inflammatory cells, most preliminary research focused primarily on their secretion of various cytokines and growth factors important for the inflammatory process including cell recruitment [135–137] and neovascularization [138]. Although macrophages in general were implicated as contributors to inflammation, a series of experiments later revealed their essential roles in bone repair. This is best exemplified in a study by Chang et al. that showed that by simply removing macrophages from primary osteoblast cultures, a 23-fold reduction in mineral deposition was observed [126]. Interestingly, in vivo depletion of OsteoMacs by various knockout systems has also been shown to markedly reduce bone formation [126,139].

Initial basic research studies identified macrophages into 2 specific cell types, classical M1 pro-inflammatory macrophages and M2 tissue resolution/wound healing macrophages. Classical pro-inflammatory stimuli in response to lipopolysaccharides (LPS) secrete a wide array of pro-inflammatory cytokines including TNF-alpha [140,141], IL-6 [142,143] and IL-1β [141,144]. M2 macrophages typically produce including transforming growth factor β (TGF-β) [145], osteopontin [146], 1,25-dihydroxyvitamin D3 [147], BMP-2 [148] and arginase, all factors implicated in tissue-repair processes [149–152]. The plasticity of macrophages suggests that their trophic role in bone tissues is highly regulated by changes to the microenvironment. While their study remains in its infancy with respect to their ability to contribute toward biomaterial integration, it remains logical to assume that under various non-physiological conditions such as low/high calcium/phosphate levels, they would be keen regulators to respond accordingly. Future studies are therefore imminently needed to better understand the role of macrophages under the above-mentioned scenarios.

Role of macrophages during heterotopic ossification in atherosclerosis and orthopedic trauma

One interesting yet rarely reported phenomenon in the bone biomaterial field are the implications of macrophages during heterotopic ossification of various pathologies. For instance, macrophages have been highly implicated in the development of atherosclerosis. Atherosclerotic plaque contains high levels of IFN-gamma, a T-helper1 cytokine that is a known inducer of the classically activated M1 macrophage. Interestingly, resident macrophages found in arteries are known to contribute to ectopic bone formation in and around vascular tissues, an area where bone should otherwise not form [133]. Similarly, heterotopic ossification is a common complication of the high-energy extremity trauma sustained in modern armed victims returning from war where some reports demonstrate as high as 60% of military combat trauma and limb amputations [130]. Pre-clinical animal models have further demonstrated that HO is precipitated in burn victims, implicating the role of inflammation in both processes [128]. In combination with these findings, it has also been shown that macrophage depletion reduces osteophyte formation in osteoarthritic models [129,131,132] and macrophages have been key players in various other bone loss disorders [153,154]. The combination of these findings has strongly suggested the implication of macrophages during heterotopic induction of bone.

One interesting finding coming from the atherosclerosis field was that it was originally thought that all macrophages involved in atherosclerotic plaque were classical M1 phenotype macrophages. However, in 2012 Oh et al. demonstrated that alternatively it was M2 macrophages that were primarily activated by endoplasmic reticulum stress [134]. This disease highlights the extreme plasticity of these cell types and ability to sense changes to the local micro-environment. While the entire mechanism driving heterotopic ossification is not fully understand, researchers now attempt to quantify inflammatory cytokines implicated in the pathogenesis of HO (such as prostaglandin E2) via either marker detection in blood or urine as a potentially useful diagnostic tool for the early detection of pathological HO [130,155] .

Conclusion

A material is osteoinductive if: (C1) it mineralizes *in vivo* (formation of a "bioactive" apatite layer on the material); (C2) it is

porous; (C3) the pores are large enough to allow blood vessel ingrowth and cell transport into the core of the material; (C4) blood supply is insufficient to keep physiological calcium and/or phosphate ion concentrations. This paradigm shift describing the osteoinductive phenomenon is not only able to address for the first time past results, but can in particular elucidate a number of unexplained results, such as why materials devoid of calcium phosphates like metals and polymers are sometimes osteoinductive, why intrinsic osteoinduction is so slow, and why ectopic bone formation happens first in the core of implanted materials. Even though the biological mechanism involved in intrinsic osteoinduction remains unclear, this new paradigm will favor the design of much more potent biomaterials by following these proposed guidelines. Interestingly, the similarities between intrinsic (material-driven) osteoinduction and trauma-related (material-free) heterotopic ossification suggest that the present paradigm could also be involved in trauma-related heterotopic ossification.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mattod.2018.10.036.

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