Growth, endocrine function and quality of life after haematopoietic stem cell transplantation

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Effect of X-irradiation on growth and the expression of parathyroid hormone-related peptide and Indian hedgehog in the tibial growth plate of the rat

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Abstract

Aim: To study the effect of irradiation on the longitudinal growth and the expression of parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (IHh) in tibial growth plates of rats.

Materials and methods: At 3 weeks of age, 30 male rats received a single fraction of irradiation (8 Gy) to their right hind limb, and small groups of animals were sacrificed 1, 2, 3, 5, 7, 10, 15, and 26 weeks after irradiation. Weight and length of both irradiated and non-irradiated tibiae were measured, and sections of the tibiae were stained with HE. PTHrP and IHh were visualized using immunohistochemical techniques.

Results: Radiation resulted in persistent growth delay of the irradiated tibiae, with a difference in length of more than 10% between the irradiated and the non-irradiated tibiae 15 weeks or more after irradiation. The growth plate architecture was disturbed, and the expression of both PTHrP and IHh was decreased in the irradiated tibiae.

Conclusion: As PTHrP and IHh are key regulators of both the pace and the synchronisation of the differentiation of growth plate chondrocytes, the reduced expression of PTHrP and IHh may contribute to the changes found after irradiation.

Introduction

In children treated for cancer, radiation has a direct effect on the epiphyses which results in disruption of growth plate architecture and contributes to the impaired growth by a yet unknown mechanism [1;2].

Many tissues show changes in the expression of regulatory proteins in response to radiation damage, a phenomenon known as 'humoral radiopathology' [3]. These humoral factors are often growth factors or other mediators of cell proliferation and differentiation (e.g., transforming growth factors, fibroblast growth factors, tumour necrosis factor, and others). Although the effects of irradiation on growth and growth plate architecture are extensively studied for over 50 years [4-8], little is known about the effects of irradiation on the expression of growth factors involved in the regulation of chondrogenesis in the epiphyseal growth plate.

Parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (Ihh) are paracrine/ autocrine factors that control the pace and synchrony of chondrocyte differentiation and are believed to co-ordinate the development of the growth plate and to influence growth rate [9].

As radiation affects architecture and growth rate of the growth plates, we were interested in its effect on the expression patterns of PTHrP and IHh. Therefore, we studied the effect of local irradiation on longitudinal growth and on the expression of both PTHrP and IHh in rat tibial growth plates.

Materials and Methods

All animal experiments were approved by a local ethical committee and performed according to Dutch law and regulation.

Irradiation

At the age of 3 weeks, 30 male Wistar rats received a single dose of X-irradiation (8.0 Gy) to the right hind limb (Philips X-ray generator, operating at 250 kV and 15 mA, equipped with a Thoraeus filter which resulted in a dose rate of 1.6 Gy/min). The left hind limb served as an internal control. Irradiation was performed at the Department of Clinical Oncology, using a setup that was

previously used for irradiation of rat gastrocnemius muscle. Details on the irradiation procedure are described in an earlier report by Hermens et al. [10]. The animals were anesthetised with a mixture of Aescoket® (50 mg/kg i.p.) and Rompun® (2 mg/kg i.p.) prior to irradiation. The right hind limbs were irradiated in posterior-anterior direction from the knee joint down. The rest of the body was protected with 2 mm thick lead plates. Special attention was given to the position of the testes, in order to prevent radiation damage. The focus–skin distance was 25 cm.

Animal Housing and Sample Collection

After irradiation, the animals were placed (2 per cage) in a light and temperature-controlled environment and were given standard laboratory chow and water ad libitum. At post-irradiation intervals of 1, 2, 3, 5, 7, and 10 weeks, groups of 4 animals were decapitated and both the irradiated and the non-irradiated tibiae were dissected and stripped. The same was done at 15 and 26 weeks with groups of 3 animals. Tibiae were weighed, and the tibial length was measured with a caliper. The tibiae were then split mid-saggittally in two equal halves and further processed for immunohistochemical analyses.

Immunohistochemistry

The detailed immunohistochemical procedures were previously described by Van der Eerden et al. [11]. The aspect of a growth plate section varies with the plane of the section (exactly craniocaudal or angulated) as well as with the position of the section (central or more peripheral in the growth plate). To ensure comparable sections, much effort is put on splitting the tibiae exactly mid-saggitally in equal halves and on the embedding and positioning of the samples on the microtome. Furthermore, only the first 15 sections of each sample were used to prevent the use of peripherally cut sections.

For PTHrP detection in the proximal tibial growth plates, the primary antibody was rabbit-derived polyclonal IgG raised against amino acids 34–53 of human PTHrP which is homologous to the PTHrP sequence in the rat (Oncogene Science, Cambridge, Mass., USA). For the detection of IHh, the primary antibody was goat-derived polyclonal IgG raised against the carboxy terminus of human IHh protein which cross-reacts with mouse and rat IHh (Santa Cruz Laboratories, Santa Cruz, Calif., USA). For optimal comparability, sections of

the irradiated and non-irradiated growth plates of animals of the same age were processed in the same experiment.

Measurements and Statistical Analyses

SPSS version 10.0 (SPSS, Chicago, Ill., USA) was used for statistical analyses. Differences in tibial length were analysed with regression models using a linear and 3rd-order curve fit. Histological measurements were done on digital micrographs of growth plate sections, using an image analysis program (Image-Pro Plus 3.0; MediaCybernetics, Silver Spring, Md., USA). We decided to use digital imaging, since blinding of the samples was not possible, due to the clearly visible differences between the irradiated and non-irradiated growth plates, making counting subjective.

In each growth plate, we measured the mean width of the individual growth plate zones, the mean height of individual columns in the proliferative zone, the amount of intervening matrix (as percentage of total growth plate area), the number of cells in the late proliferative and early hypertrophic zone, and the number of PTHrP-positive and IHh-positive cells in this 'transitional' zone. In individual animals, the results of the irradiated growth plate were compared to those of the normal growth plate. As there were only 3 or 4 animals at each time point, the animals were then clustered into three age groups: young (1–3 weeks after irradiation), middle-aged (5–10 weeks after irradiation), and old (15–26 weeks after irradiation). Differences between irradiated and non-irradiated growth plates were analysed in each age group using A Wilcoxon signed-rank test.

Results

There were no visible effects of the irradiation in any of the animals, i.e., we did not observe functional impairment or skin lesions of the irradiated hind limb. In all animals, the irradiated tibia was shorter as compared with the non-irradiated tibia. This was noticed already 1 week after irradiation. Furthermore, the difference increased with increasing post-irradiation intervals, suggesting continuous growth delay (figure 1a).

On histological examination, a clear disruption of the growth plate architecture was found at all times after irradiation (figure 2). The columns were less straight and less parallel to each other, and many columns did not extend

across the entire growth plate. The mean reduction in column height in the proliferative zone was 38% in the young animals (p = 0.002), 27% in the middle-aged animals (p = 0.04), and 13% in old animals (not significant). Furthermore, there were some clusters of cells that were not organised in columns, as well as clustered columns. The amount of intervening matrix was increased in the middle-aged (mean increase 26%; p = 0.03) and older animals (mean increase 17%; p = 0.04), but not in the younger animals. There were some cells with a hypertrophic appearance within the proliferative zone (see arrows in figures 2b and d) and some columns failed to complete the transformation from cartilage to bone, which resulted in cartilage islands within the trabecular bone (see arrowheads in figures 2f and 3f).

Due to these structural changes, the different zones (i.e., resting, proliferative, hypertrophic, and calcifying zones) were less well defined, making it difficult to establish the exact width of each zone. We could not establish significant changes in the growth plate width nor in the width of individual zones (data not shown).

Both the absolute and relative numbers of PTHrP-positive cells in the irradiated growth plates were reduced (figure 3a–f). This reduction was seen already 1 week after irradiation and did not restore with increasing time interval after irradiation. The reduction was seen in both the stem cell zone and (most prominent) in late proliferating and early hypertrophic chondrocytes which were previously shown to express PTHrP [11]. The mean relative reduction in PTHrP-positive cells in the 'transitional' zone was 52% in the young animals (p = 0.02), 43% in the middle-aged animals (p = 0.04), and 36% in the old animals (p = 0.05). We saw a similar reduction in the number of IHh-positive cells in the irradiated tibiae (figure 3g–h). Since overall staining of IHh was very weak, computer-aided digital imaging and quantification turned out to be unreliable.

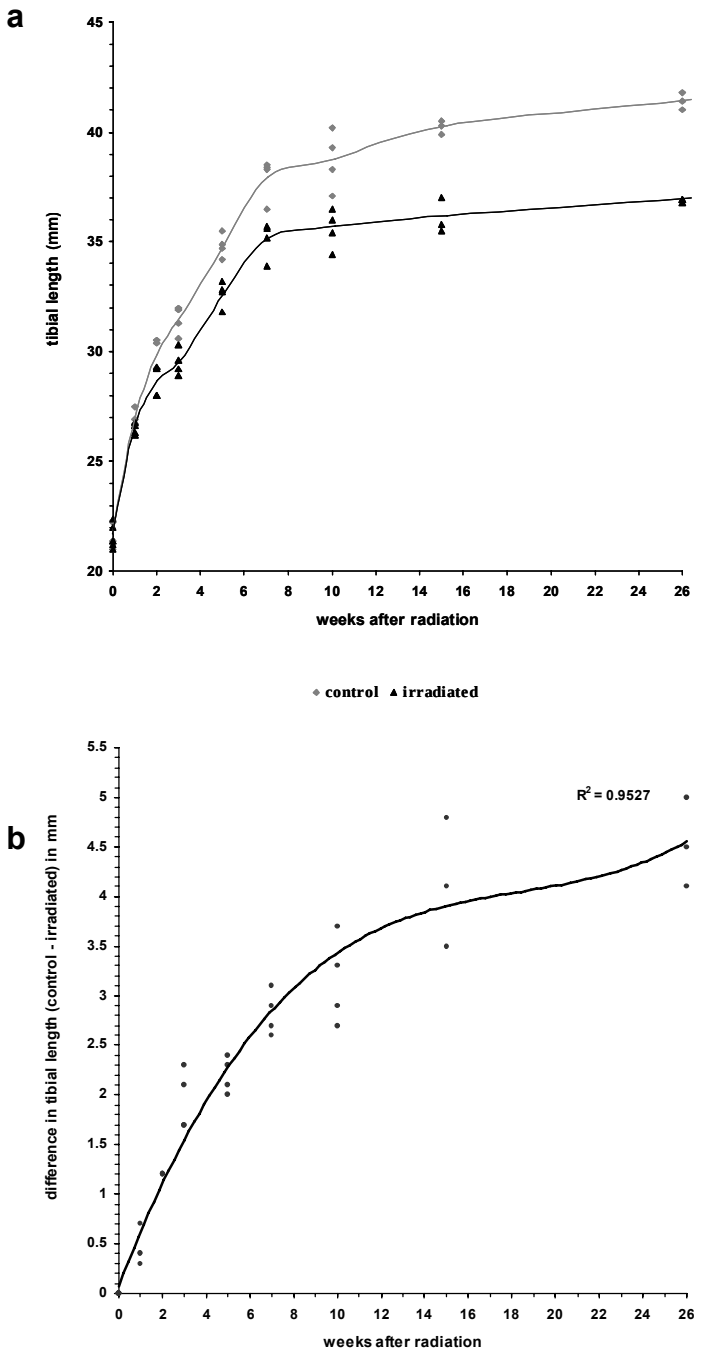

Figure 1. **a.** Tibial length of irradiated (▲) and non-irradiated (♦) legs 0 to 26 weeks after irradiation. **b.** Individual differences in tibial length (control minus irradiated tibia) versus weeks after irradiation.

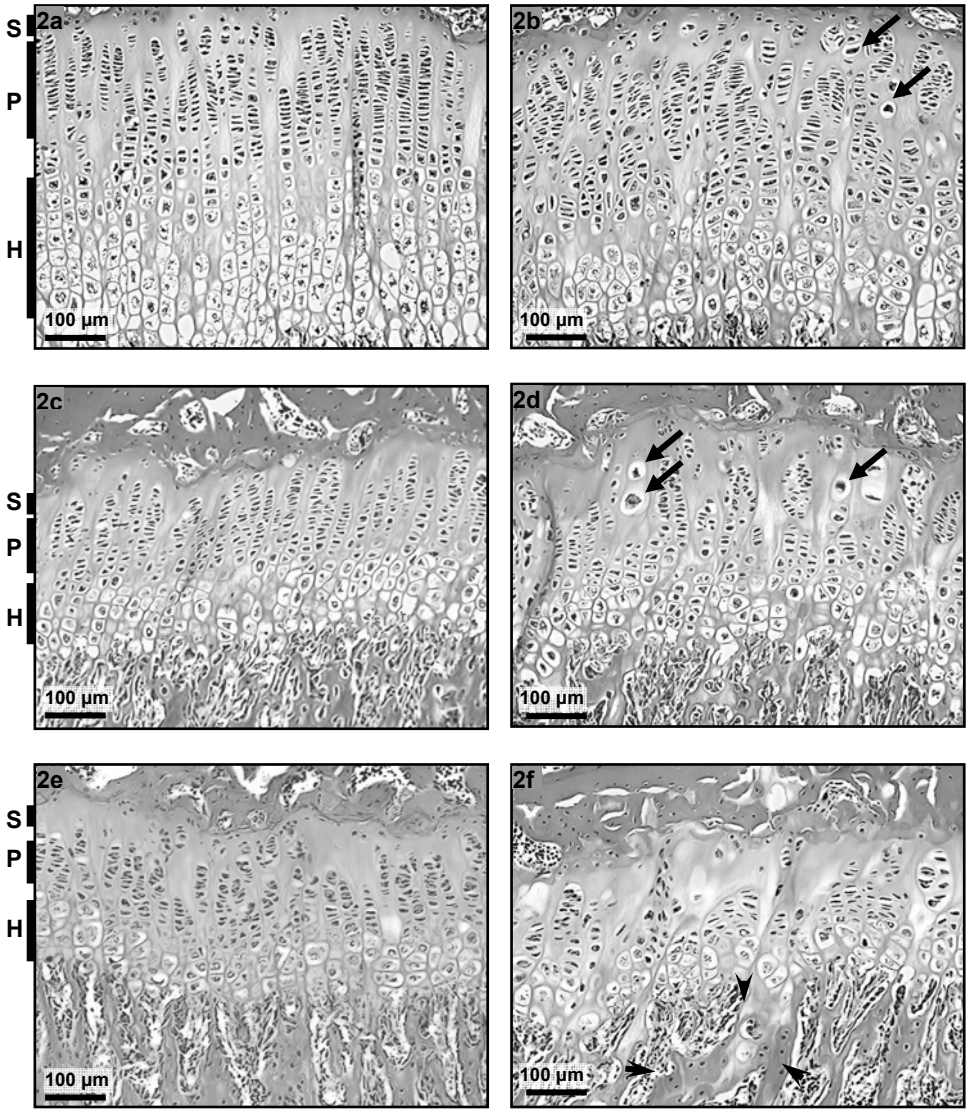

Figure 2. Morphological changes after irradiation.
Irradiated tibiae are presented on the right-hand side. HE staining. ***a,b*** 2 weeks after irradiation. ***c,d*** 7 weeks after irradiation. ***e,f*** 15 weeks after irradiation. Note the disorganisation of the growth plate and the presence of hypertrophic cells (arrows in b and d) in the proliferative zone and the cartilage islands in metaphyseal bone (arrowheads in f). S = Stem cell zone; P = zone of proliferation; H = zone of hypertrophy.

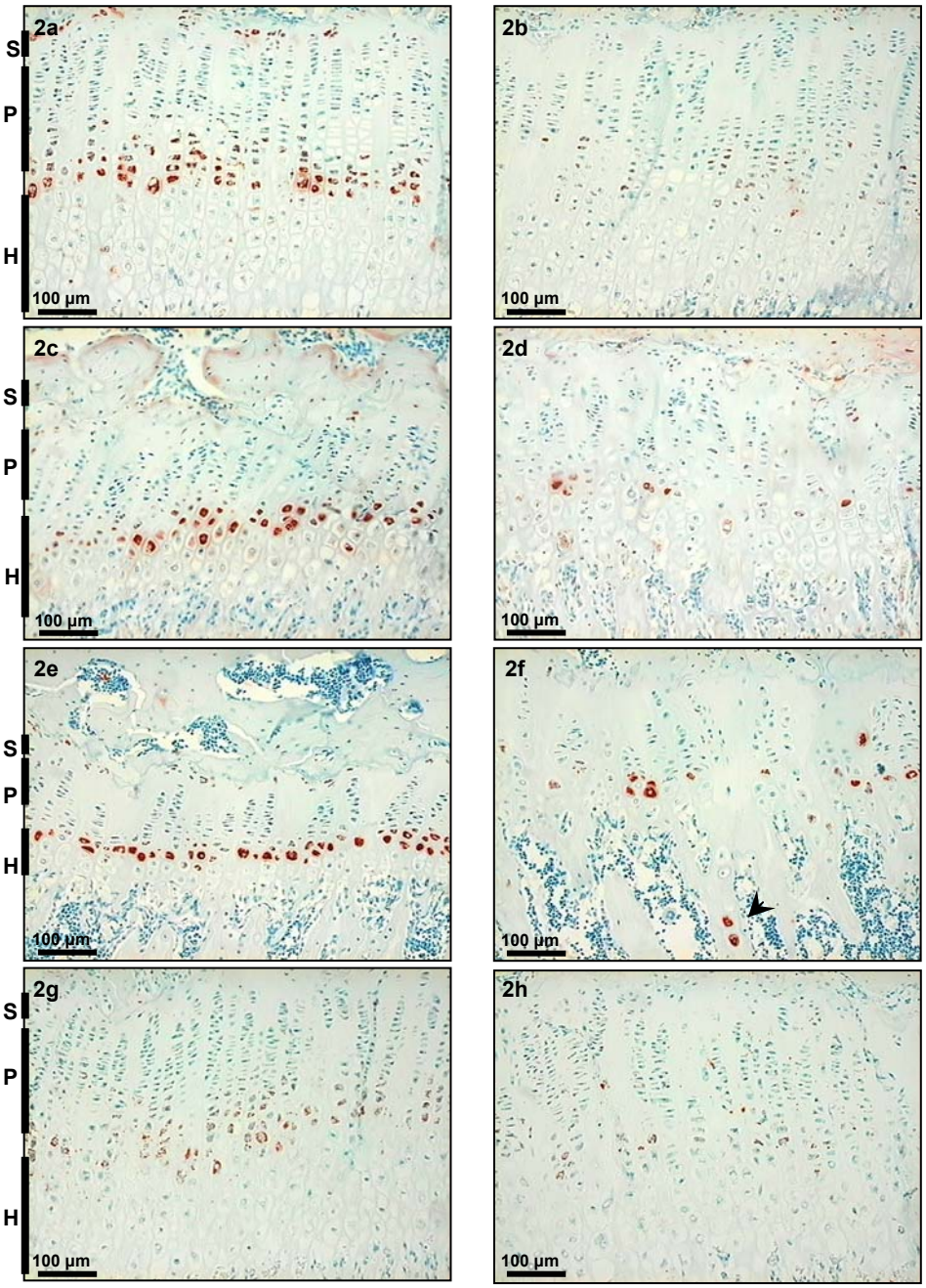

Figure 3. Presence of PTHrP and IHh in the tibial growth plates at different times after irradiation. Irradiated tibiae are presented on the right-hand side. PTHrP and IHh levels are clearly reduced after irradiation. **a,b** PTHrP 2 weeks after irradiation. **c,d** PTHrP 7 weeks after irradiation. **e,f** PTHrP 15 weeks after irradiation; note the islands of hypertrophic cells (with PTHrP staining) within the bone (arrowheads in f). **g,h** IHh 2 weeks after irradiation. **S** = Stem cell zone; **P** = zone of proliferation; **H** = zone of hypertrophy.

Discussion

In our clinic, many haematopoietic stem cell transplant recipients who are treated for haematological malignancies show growth delay after a single fraction of 8.0 Gy of total-body irradiation [12]. As this radiation dose is known to damage the growth plate architecture in many species, including rats [8;13], we decided to use this dose for our experiments. As could be expected, a single dose of 8.0 Gy to the right hind limb resulted in structural as well as functional damage (growth delay) to the epiphyseal growth plate. The difference in tibial length between the irradiated and non-irradiated limb increased during the whole follow-up period, suggesting that growth delay was persistent, and there was certainly no catch-up growth. This is in line with the human situation, where damage to the growth plates also results in persistent growth retardation.

The increase in intervening matrix in the middle-aged and old animals suggests that growth retardation could be the result of exhaustion of germinal cells after radiation-induced cell death in the stem cell zone. The decreases in PTHrP and IHh, however, suggest that changes in paracrine/autocrine factors could also contribute to both growth delay and structural changes. PTHrP is a paracrine/autocrine factor, produced in most cell types in the body. Its functions include the regulation of cell cycle, differentiation, apoptosis, and developmental events [14]. In prenatal growth plates, it delays the transition of chondrocytes from a proliferative towards a hypertrophic state and synchronises the rate of differentiation in the growth plate [15]. If PTHrP is over-expressed, the transition from proliferation to differentiation is impaired, resulting in prolonged proliferation and delayed differentiation [16]. In the absence of PTHrP, however, this transition is accelerated, which leads to premature differentiation and growth delay [17]. Therefore, both the PTHrP and the PTH/PTHrP-receptor-deficient mice show accelerated hypertrophy and mineralization in the cartilage. Furthermore, PTH/PTHrP receptor knockout mice show delayed vascular invasion, whereas the double homozygous PTHrP and PTH/PTHrP receptor knockout mice do not. Thus, PTHrP must slow vascular invasion by a mechanism independent of the PTH/PTHrP receptor [18]. PTHrP expression is stimulated by IHh which is expressed in early hypertrophic chondrocytes [19]. An increase in PTHrP slows down differentiation and results in a reduction of IHh-producing cells, thus forming a negative

feedback loop which co-ordinates the development of the growth plate and influences growth rate [20]. All components of this feedback loop (i.e., PTHrP, IHh, and their respective receptors) are also present in the post-natal growth plate of the rat [11], and the feedback loop is, therefore, supposed to be functional after birth as well.

A recently published in vitro study on irradiated avian growth plate chondrocytes [21] describes a dose-dependent decrease in both PTHrP mRNA and PTHrP protein (but not other autocrine and paracrine factors) 24 h after irradiation which was related to a radiation-induced increase in cytosolic calcium. In addition to these findings, we found in our in vivo experiments that PTHrP and IHh continue to be reduced after longer post-irradiation intervals (up to 26 weeks). Furthermore, radiation resulted in growth delay and disorganisation of the columnar structure of the growth plate, which could indicate impaired synchronisation of the processes of proliferation and differentiation in the growth plate. As PTHrP and IHh play a key regulatory role in these processes, it is not unlikely that the radiation-induced disturbances in growth plate differentiation are related to the changes in PTHrP and/or IHh expression we found after irradiation. In normal growth plates, however, premature differentiation mediated by a decrease in PTHrP expression would not only result in growth delay, but also in accelerated differentiation and increased bone formation, something we did not see in our experiment. This implies that differentiation is also impaired. Indeed, in vitro experiments in other species have shown a decrease in matrix production and mineralization after irradiation [22]. There are several possible explanations for the reduced expression of PTHrP we found after irradiation: (1) irradiation may have disturbed the proliferation of chondrocytes which forces them into differentiation, achieved by a reduction in PTHrP expression, and (2) irradiation has impaired the differentiation of chondrocytes, and the decrease in PTHrP expression is an attempt to overcome this. Both explanations imply that decreased PTHrP expression is a regulatory mechanism, but they do not explain why the decreased expression of PTHrP did not increase IHh expression, as could be expected, if the negative feedback loop is present in postnatal growth plates. A third possibility is that the reduction in the expression of both PTHrP and IHh is just a consequence of the impaired function of differentiating chondrocytes, as (at least in other species) other

differentiation markers are also reduced after irradiation (e.g., alkaline phosphatase and collagen X) [22].

Whatever the mechanism, however, a reduction in PTHrP and IHh is expected to have an effect on growth and differentiation. We, therefore, conclude that a reduced expression of both PTHrP and IHh may contribute to the disturbances in growth and growth plate architecture seen in rats after irradiation.

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