An organotypical *in vitro* model for vascular tissue remodelling and its application to study radiation effects

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Abstract

An organotypic *in vitro* model, to study vascular tissue remodeling, was evaluated as a function of culture period. In order to validate the model as a tool for studying vascular responses to damage, a dose-response analysis to ionizing irradiation was included. Rat aortic rings were explanted *in vitro* after being irradiated with single doses of ⁶⁰Co γ -rays, namely 0, 5, 10, 15, 20 or 25 Gy. Irradiated and sham-irradiated aortic rings were cultured for 3 weeks. Explant outgrowth on an adhesive substrate was evaluated by macroscopical scoring, and rings derived from each irradiation group together with the outgrowths were fixed and embedded in paraffin after 2, 7, 14 and 21 days. Bromodeoxyuridine incorporation, α smooth muscle actin and collagen types I and III were scored on immunohistochemically stained sections. For each studied parameter, irradiated and sham-irradiated rings were compared. In cultures of sham-irradiated rings, alterations from a contractile towards a synthetic/migratory smooth muscle cell phenotype were confirmed. After 3 weeks, fullgrown cultures had formed. Irradiation slowed down the phenotypical modifications. After 15 Gy, irradiation explant outgrowth was already retarded; after 25 Gy, the outgrowth was completely blocked. On the other hand, a dose of 15 Gy or more induced an increased collagen I production in the tunica media. In conclusion, the present organotypical *in vitro* model fits to analyse dynamics in the original vascular tissues as well as in the primary outgrowth. It enables to confirm features of tissular reorganization and effects of ionizing radiation described *in vivo*.

Abbreviations: α SMC actin, α smooth muscle actin; BrdU, Bromodeoxyuridine; coll I, collagen type I; coll III, collagen type III; ECM, extracellular matrix; FCS, Foetal calf serum; VSMC, vascular SMC.

Introduction

The vascular wall is not static, as the vascular components – cells and extracellular matrix (ECM) – dynamically increase, decrease and/or reorganize in response to physiological and pathological stimuli (Dubey et al., 1997).

Damage to the arterial wall stimulates cells to proliferate, migrate and elaborate ECM. This may result in remodeling, plaque and/or neointimal growth (King, 1996; Schwartz and Holmes, 1996).

The mechanisms regulating interactions between inhibitory and promoting factors for growth and matrix production in the vascular wall are still not well understood, as recently reviewed by Dubey et al. (1997).

In vivo, many growth promoters and growth inhibitors are present, so that *in vitro* observation of interactions may represent a simplification.

Moreover, similar morphological features of vascular tissues are observed in pathological and *in vitro* conditions (Schürch et al., 1997). In an attempt to

analyse possible interactions between growth stimulators and growth inhibitors, several *in vitro* models have been applied (Hultgardh-Nilsson et al., 1997; Miyazawa et al. 1997; Myers and Tanner, 1998; Stevenson and Lange, 1997; Wills et al., 1996; Vargas et al., 1993). Explant-derived cells may provide a suitable study model which mimics the pathological state within intimal lesions: To be cultured they are selected according to their capability of migrating out of the tissue segment (Brenner et al., 1996).

Furthermore, appropriate cellular reactions to noxal stress, including radiation insults, depend on the integrity of the ECM. According to Stevenson and Lange (1997), the ECM is determined, in turn, by interactions among the cells themselves, by interactions between cells and matrix. Therefore, in the present study variation of parameters were examined both in the cells growing out of the tissue segment and in the original organotypical tissue fragments. In an attempt to analyse reactions of vascular smooth muscle cells to noxal stress, cellular outgrowth as well as cell division, together with variations of cellular phenotype and matrix were observed in explant cultures starting from control adult rat aortic rings and rings derived from irradiated aortic segments.

This application is chosen because of the numerous trials on the use of intracoronary irradiation for the prevention of restenosis.

Materials and methods

In the present *in vitro* investigation, aortic rings and their explants were studied. Immunohistochemical methods were applied to visualize a number of cellular and extracellular markers. As well effects of the *in vitro* technique as of the irradiation are evaluated.

Aortic segments

Six rats (Wistar, male, ± 300 g) were killed by cervical dislocation, the thoracic aorta was removed and rinsed twice in Ringer's solution containing antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin). Further manipulations were performed in sterile conditions. Before irradiation, the aortae were freed from adhering fat and loose connective tissue and placed in a closed tube (Falcon, Becton Dickinson Labware, Franklin Lakes NJ, U.S.A.) containing culture medium at 37 °C.

Irradiation

One aorta was sham irradiated, the others were subjected to acute single ^{60}Co γ -irradiations of 5, 10, 15, 20 or 25 Gy. Dose rate was 1 Gy/min. During irradiation the tubes, containing the aortic fragments, were kept in a water bath at 37 °C.

Organotypical cultures

Directly after irradiation or sham irradiation, 1 mm aortic rings were cut. Four petri dishes (diameter 3.5 mm), each containing at least 3 rings, were prepared per aorta. Foetal calf serum (FCS, 0.5 ml per dish, Life Technologies, Merelbeke, Belgium) was added, and the cultures were kept at 37 °C overnight. Thereafter, FCS was replaced by Minimal Essential Medium containing Earle's salts (Life Technologies), 15% FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. The dishes were placed at 37 °C in a humidified atmosphere (5% CO_2 /95% air) during 3 weeks and fed every 3–4 days with fresh culture medium.

Explant outgrowth

Explant outgrowth was evaluated by semiquantitative scoring at regular intervals (on days 2, 5, 7, 10, 14, 18 and 21) by means of an inverted microscope equipped with phase contrast. The scoring system used is illustrated in Figure 1

Bromodeoxyuridine labeling and fixation

Bromodeoxyuridine (BrdU) incorporation identifies the cells entering S-phase during the labeling period preceding the fixation of the aortic rings. A 24-h labeling was started after 1, 6, 13 and 20 days of incubation *in vitro*. Hereto, the medium of 1 dish per radiation dose was replaced by medium containing 10^{-4} M BrdU/ml (Sigma Chemical Company, St. Louis, Mo). After labeling (i.e. at 2, 7, 14 and 21 days, respectively called t2, t7, t14 and t21 series) the rings were rinsed 3 times in BrdU-free medium and fixed in 4% buffered (pH 7.4) paraformaldehyde.

Histology

After fixation, the rings were dehydrated in graded ethanols and embedded in paraffin. Sections (5 μ m) were cut longitudinally, as to allow the study of both the outgrowth, located at the former contactzone with the dish, and the aortic ring itself (see Figures 2B

Figure 1. Representation of the scoring of explant outgrowth: A: aortic ring. Score 0: no explant outgrowth. Score +: outgrowth of solitary cells. Score ++: a few small patches of primary outgrowth (see also picture taken with inverted microscope). Score +++: many patches of primary outgrowth. Score ++++: many large patches of primary outgrowth, dish not completely covered (see also picture taken with inverted microscope). Full grown: the petri dish is completely covered by the outgrowth.

and 2E). The sections were mounted on organosilan coated slides. Before staining, sections were rehyd-

rated in ethanols. Eosine-Mayer's hemalum staining was performed for routine evaluation of the sections.

Figure 2. (Immuno)histochemical stainings on longitudinal 6 μm paraffin sections of cultured vessel rings and outgrowth, if present: A: HE staining after fixation at t7 following 5 Gy irradiation. Remark the explant covering the entire aortic segment. B: HE staining after fixation at t21 following sham irradiation, showing a large multilayered explant outgrowth. C: Alfa actin staining after fixation at t2 following sham irradiation, showing intense staining in the t. media and in vasa vasorum. D: Alfa actin staining after fixation at t14 following 5 Gy irradiation. Remark the lowered positivity in the t. media and the presence of alfa actin in the outgrowth. E: Coll I staining after fixation at t21 following sham irradiation with almost complete loss of positivity in the t. media and strong positivity in the explant outgrowth. F: Coll I staining after fixation at t21 following 20 Gy irradiation with preservation of positivity in t. media.

Immunohistochemistry

For immunohistochemistry polyclonal antibodies raised against rat collagen type I (coll I, dilution 1/100) and against collagen type III (coll III, dilution 1/500) were purchased from Novotec (Lyon, France). Both antibodies bind to epitopes on cellular and extracellular coll I or coll III, respectively. Monoclonal antibodies were used in order to visualize BrdU (dilution 1/500, Novocastra, Newcastle U.K.) incorporation or α smooth muscle actin (α SMC actin, dilution 1/200, Dako, Glostrup, Denmark).

Specificity of all antibodies was demonstrated using positive and negative controls. The distribution of BrdU, α SMC actin, coll I or coll III was investigated using a biotinylated secondary antibody (swine anti rabbit or rabbit anti mouse, Dako, Glostrup, Denmark), followed by streptavidine peroxidase and visualisation by diaminobenzidine tetrahydrochloride. Briefly, endogenous peroxidase was blocked by 3% H_2O_2 for 10 min at room temperature and nonspecific antibody staining was blocked by incubation with 5% normal rabbit or normal swine serum in phosphate buffered saline containing 1% bovine serum albumin and 0.02% Tween 20 for 30 min at room temperature. All antibodies were diluted in phosphate buffered saline containing 10% blocking serum.

The polyclonal coll I and coll III antibodies applied in the present study, are raised against cellular and extracellular epitopes, which means that apart from the extracellular collagens fibers, proto- and/or procollagen molecules are also visualised in the tissue sections.

In the BrdU staining protocol, permeabilisation was performed (0.1% trypsin and 0.1% $CaCl_2$ in 50 mM Tris-buffer) after endogenous peroxidase blocking and a tyramide signal amplification step was included after application of the secondary antibody. Hereto, a NEN (Boston, MA) kit containing biotinyl tyramide reagent, blocking reagent, amplification diluent and streptavidine horseradish peroxidase was purchased.

After immunostaining, the different parameters were semiquantitatively scored during lightmicroscopical evaluation. The score for BrdU (evaluation of positive cells) or for α SMC actin (evaluation of staining intensity and distribution) was noted after observing at least 2 sections per experimental group, while the following scores were blindly applied: 0, +, ++, +++ or ++++.

Per experimental group, 2 coll I and 2 coll III

stained sections were scored (score 0, 1, 2, ... to 6, as an representation of staining intensity and distribution). Collagen was scored in t. media and t. adventitia. The highest score (score 6) was found in the t. adventitia after coll I staining.

Results

Explant outgrowth

The scores for explant outgrowth, as visualized using an inverted microscope, are represented in Table 1A. Already 5 days after initiating the cultures, cellular outgrowth is observed. In the dishes containing non irradiated artery segments, this outgrowth is very rapidly increasing and results in complete covering of the culture dish at t21.

The 5 Gy and 10 Gy cultures behave as the sham-irradiated ones, apart from some lower scores at t10 and t14.

Explant outgrowth after irradiations of 15 Gy or more is decreased as compared to 0 Gy cultures, at least during the studied incubation period. When rings are subjected to 15 Gy, the explant does not cover the culture dish at the end of the studied period. While after 20 Gy still a few solitary cells are found on the culture dish, no outgrowth at all was observed in dishes containing 25 Gy irradiated rings.

Histological sections show that when outgrowth is present, it is not limited to the petri-dish surface, but also covering the aortic ring (Figure 2A).

BrdU incorporation

To evaluate DNA synthesis and consequently mitotic activity during the considered culture period, BrdU incorporation was visualized. In Table 1C, the scores for BrdU incorporation in the tunica media, as observed by an immunohistochemical staining, are shown. BrdU incorporations were found in all studied rings from 7 days incubation onwards, independent of the irradiation dose to which they had been exposed before the *in vitro* setup. The numbers of cells, labeled during a 24 h period, were low but clearly visualized.

In the explant outgrowth, BrdU incorporation is directly related to the outgrowth intensity (compare Table 1A with Table 1D). Only in some rings at t7 and t14, adventitial fibroblasts positive for BrdU, have been observed (Table 1E).

Table 1. A: Results of macroscopical scoring of explant outgrowth as studied by means of phase contrast microscopy. B: Results of immunohistochemical evaluation of α smc actin in the t. media. C: Results of immunohistochemical evaluation of BrdU incorporation in the t. media. D: Results of immunohistochemical evaluation of BrdU incorporation in the outgrowth. E: Results of immunohistochemical evaluation of BrdU incorporation in the t. adventitia

Dose (Gy)	Days of culturing						
	2	5	7	10	14	18	21
A: Explant outgrowth							
0	0	+	+	+++	++++	++++	full grown
5	0	+	+	++	++++	++++	full grown
10	0	+	+	+	+++	++++	full grown
15	0	+	+	+	++	++	+++
20	0	0	0	+	+	+	+
25	0	0	0	0	0	0	0
B: Alfa actin in t. media							
0	+++		++		+		+
5	++++		+++		+		+
10	+++		+++		+		+
15	++++		++		++		++
20	+++		+++		+		++
25	+++		++		++		++
C: BRDU incorporation in t. media							
0	0		++		+		+
5	0		++		+		+
10	0		+		++		+
15	0		+		++		+
20	0		+		+		+
25	0		+		+		0
D: BrdU incorporation in outgrowth							
0	/		+++		+++		+++
5	/		++		++		++
10	/		++		++		++
15	/		+		+		++
20	/		/		+		+
25	/		/		/		/
E: BrdU incorporation in t. adventitia							
0	0		+		0		0
5	0		0		+		0
10	0		+		0		0
15	0		+		+		0
20	0		+		0		0
25	0		0		0		0

Alfa smooth muscle cell actin positivity

α SMC actin was visualized as a phenotype marker for smooth muscle cells. In the adventitia, staining is only found in SMC of the vasa vasorum (Figure 2C). In all conditions where outgrowth was present, a faint α SMC actin positivity could be observed (Figure 2D).

Scores for α SMC actin distribution in the t. media, in the different experimental conditions, are represented in Table 1B. Initially (t2) most of the SMC in the t. media are α SMC actin positive: In t2 and t7 cultures no major difference was found between irradiated and non-irradiated rings. After 14 and 21 days, however, the scores might be divided in 2 groups: low scores are noted for non-irradiated as well as 5 or 10 Gy irradiated rings, while the scores after 15, 20 or 25 Gy irradiation are generally higher.

Collagen type I and collagen type III positivity

Coll I and coll III were visualized as important ECM components. In the t. adventitia of control and irradiated vessel fragments high scores (scores 6) for coll I are noted, independent of irradiation dose or culture period (Figure 2F). In each aortic ring adventitial coll III was scored very low (scores 1). If explant outgrowth is present, it exhibits as well coll I (Figure 2E) as coll III immunostaining, distributed over its complete surface. The immunostaining in the explants did not vary according to culture period nor to preceding irradiation dose.

Results of coll I and coll III scores in the t. media are shown in Figures 3A and 3B, respectively.

The t. media of sham irradiated rings exhibit a decrease of the coll I scores as the culture period proceeds. The scores of the 5 and 10 Gy irradiated rings point towards a less pronounced decrease along with the incubation time. After an irradiation of 15 Gy or more, coll I scores increase after 3 weeks of culture. This is opposed to the sham irradiated and low dose (5–10 Gy) irradiated rings.

The scores of coll III in t. media of non-irradiated rings represent a decrease which is also directly proportional to the culture period. After 10 and 15 Gy irradiation no coll III was observed from t14 onwards. Except for the 20 Gy series, at t21 no coll III was visualised in the sections derived from irradiated rings.

Discussion

In order to assess reorganization in the vascular wall, several types of *in vitro* models have been applied, e.g. vascular smooth muscle cells (VSMC) cultures (Hultgardh-Nilsson et al., 1997; Miyazawa et al., 1997), co-cultures of endothelial cells and VSMC (Myers and Tanner, 1998), fibroblast-like cells in 3-dimensional gel structure (Stevenson and Lange, 1997) or explant-derived VSMC (Vargas et al., 1993; Wills et al., 1996).

At present, an organotypical explant model is described which enables us to analyse modifications in both the original segment of the vascular wall, as well as in the primary outgrowth. Cellular outgrowth, DNA synthesis, cell division, some phenotypical cellular changes and ECM alterations were evaluated during a 3 week lasting culture period.

The SMC in adult arteries are known to be 'quiescent', non-dividing cells (Owens, 1995; Dubey et al., 1997). As reviewed by Owens (1995) there exists a continuum of VSMC phenotypes, ranging from a developmentally immature but committed SMC that proliferates rapidly, expresses low levels of smooth muscle contractile proteins, towards the fully differentiated SMC that shows a low proliferative rate and is a cell geared almost exclusively to contraction. In order to contract, SMC in healthy, fully developed blood vessels are characterized by a predominance of the α SMC actin isoform, as opposed to SMC from respiratory, gastrointestinal and genitourinary tracts. Only 1% of the cells in the adult aortic media are α SMC actin negative, as opposed to early months of life, where 50% of the cells lack α SMC actin (Schürch et al., 1997).

Dubey et al. (1997) focused on the complex mechanisms which may interact with adult VSMC to induce proliferation, migration and ECM synthesis. Once these features are expressed, they are called myofibroblasts (Schürch et al., 1997). Myofibroblasts are, amongst others, described in responses to injury and repair phenomena of which atherosclerotic lesions are an example (Schürch et al., 1997). This implies that during modulation from contractile to synthetic/migratory phenotype there is a loss of α actin (Schwartz et al., 1995; Carmeliet and Collen, 1998).

As there is little evidence that there are subtypes of VSMC's in adult animals that subserve primarily a synthetic/migratory role under normal conditions, myofibroblasts observed after vascular injury seem to

Figure 3. A: Bar diagram, representing the means of 2 scores for collagen type I in t. media of sham-irradiated (0 Gy) or gamma irradiated (5, 10, 15, 20 or 20 Gy) aortic rings, cultured for 2, 7, 14 or 21 days. B: Bar diagram, representing the means of 2 scores for collagen type III in t. media of sham-irradiated (0 Gy) or gamma irradiated (5, 10, 15, 20 or 20 Gy) aortic rings, cultured for 2, 7, 14 or 21 days.

be derived from fully contractile VSMC's (Owens, 1995).

In our laboratory, previous *in vivo* intraperitoneal BrdU injections into male adult rats (same weight, same strain as in present study) did not show positivity in the aortic t. media. This was opposed to simultaneously studied proliferative tissues from the same animals, like tubular cells in post-ischemic kidneys. In the same conditions, a general positivity for α SMC actin was detected in the aortic t. media (unpublished results).

The ECM in blood vessels contains coll I and coll III. Whereas coll III is quantitatively more important during fetal development, coll I is more abundant in the adult. The organic matrix of a plaque also consists mainly of coll I and coll III (Carmeliet and Collen, 1998). Therefore the distribution and density of these two types of collagen was studied.

In the present study all cultures were started from normal thoracic aortic rings, prepared from adult rats. It has already been concluded by others (Gajdusek et al., 1995; Hultgardh-Nilsson et al., 1997) that phenotypic alteration from contractile to synthetic – like in the formation of atherosclerotic and restenotic lesions – also occurs when SMC are grown in culture.

Our findings are in agreement with these previous observations. In the culture dishes containing non-irradiated vessel rings, indications of a modified phenotype were observed. The first explant outgrowth was observed after 5 days. From t7 onwards, BrdU incorporations were visualised in the aortic fragment and in the outgrowth. This is in accordance with data obtained in rat and rabbit models of post-angioplasty restenosis: cell proliferations begin in the media within 48 h after injury and migration is observed after a week (Wilcox et al., 1996). Later on (from t14 onwards), the lower α actin positivity in the tunica media and the increased outgrowth in our control cultures further point towards the presence of proliferative and migratory SMCs. In the control cultures it was found that coll I as well as coll III scores decrease as time proceeds.

Furthermore, once the explant outgrowth has started, SMC produce their own growth factors, e.g. platelet derived growth factor, a very potent stimulator of both VSMC migration and proliferation (Hultgardh-Nilsson et al., 1997; Rubin et al., 1998). Also coll I is known to stimulate migration (Zetter and Brightman, 1990; Nagler et al., 1997; Shannon et al., 1997; Bode et al., 1999). The latter fact does not seem to fit with the present findings as we found declining scores of

coll I simultaneously with the steep increase of explant outgrowth in control cultures.

In earlier *in vitro* studies primary rat aortic SMC were obtained after enzymatic treatment of arterial fragments (Hultgardh-Nilsson et al., 1997) or using passages 2–5 of explants (Gajdusek et al., 1995). The present findings, starting from 'quiescent' SMC may supply a model which is even more close to the *in vivo* situation. Thus, the present *in vitro* procedure in combination with immunohistochemical techniques enables one to observe architectural alterations, migration and proliferation induced by disequibrated environmental conditions. The latter *in vitro* system might to a certain extent be comparable to pathological situations, like atherosclerosis and restenosis *in vivo*.

At the moment, endovascular brachytherapy using gamma or beta emitters is one of the techniques applied to prevent post-angioplasty restenosis (Wiedermann et al., 1994; Hall et al., 1996; Condado et al., 1997; Teirstein et al., 1997; King et al., 1998; Serruys and Carlier, 1999). In order to further validate the model as a tool for studying vascular responses to damage, a dose-response analysis to gamma irradiation was included. Therefore, after irradiation of the starting material, namely the freshly prepared aorta, cultures were kept in the same conditions as control sham-irradiated ones. Responses to the different irradiation doses at the level of the analysed cellular and extracellular parameters were analogously studied along the culture period. At each time point, these responses were compared to the ones observed in the sham-irradiated rings, since the latter represent phenomena purely as a result of culture conditions. By doing so, one can discern effects that can be attributed explicitly to irradiation.

We may accept that the SMC in the aortic fragments are still of the 'quiescent' phenotype at the moment of gamma irradiation, since the latter intervention occurred within one hour following dissection.

Irradiation of 25 Gy completely blocked explant outgrowth. After 20 Gy a few cells were observed; after 15 Gy outgrowth was already retarded. Doses of 10 or 5 Gy did not show substantial difference as compared to the sham-irradiated vessel rings.

These data can be correlated to the immunohistological observations made on tissue sections of these organotypical cultures. Alfa SMC actin scores in the t. media are inversely related to the explant outgrowth. This could indicate that gamma irradiation inhibits the switch to a synthetic phenotype. On the other hand, after 20 Gy and even in rings exposed to 25 Gy some

BrdU positive cells could still be found, coll III synthesis was not completely stopped and the coll I score even showed an increase in the 3 week old cultures. These findings indicate that (at least some) cells in the t. media of 20 or 25 Gy irradiated rings are still metabolically active.

These dose-responses fit well with findings in literature, both in animal studies and in *in vitro* studies after acute irradiations (Shäfer et al., 1998; Crocker, 1996; Waksman, 1996; Brenner et al., 1996; Serruys and Carlier, 1999). It has been concluded that irradiation of vascular tissues with doses up to ± 20 Gy is not able to stop the restenosis process, but most likely will result in a growth delay (Brenner et al., 1996). Wiedermann et al. (1994) also found acute functional SMC impairments (i.e. endothelium independent vasodilatation) after a minimal dose of 15 Gy gamma irradiation *in vivo*. Lower doses (± 10 Gy or lower) are known to have no effect. In some studies these low doses are even found to be stimulatory (Weinberger and Simon, 1997).

It is often suggested that the ability of ionizing radiation to halt cell growth is primarily by damaging the DNA of dividing cells e.g. to treat malignant and nonmalignant proliferative conditions. In that respect, some consider neointima formation as a benign proliferative condition (Weinberger and Simon, 1997).

Consequently, is it unlikely that the halted outgrowth in our cultures subjected to irradiation is due to damage of dividing SMC, since they were not dividing at the moment of irradiation. This condition is opposed to most of the known *in vivo* and *in vitro* models. The former are irradiated after the initiation of vascular damage (extention, mechanical damage ...), and thus after triggering proliferation. In the latter, irradiated target cells or tissue fragments have been cultured before irradiation, and so doing they were already proliferative (e.g. SMC cultures as primary explants or at later passages, 3 d gel models, co-cultures). This would imply that in our experimental setting, irradiation did not stop migration and proliferation, but retarded its initiation. In that respect, irradiation induced damage to cell motility could be of importance, a hypothesis which needs to be explored in a further study. The delay of a phenotypical alteration by ionizing radiation could lead to a maintained relaxation-contraction response.

In conclusion, apart of being a tool for studying alterations of the cellular and extracellular components of the vessel wall and cell migration capability, the organotypical culture system described at present also

fits to study effects of ionizing irradiation. Whether this *in vitro* model is appropriate to analyse effects of biochemical or pharmacological agents on the aforementioned properties of vascular tissues as well, needs to be further elucidated.

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