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TECHNICAL NOTE

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Immunohistochemical Expression of HIF-1α in Response to Early Myocardial Ischemia

ABSTRACT: This study aims to evaluate the effects of ischemia on the myocardial fibers and the expression of the transcriptional factor for angiogenesis hypoxia-inducible factor-1 alpha (HIF-1α) in human heart specimens. We have prospectively analyzed the HIF-1α expression in human ischemic hearts with the ABC-inmunohistochemistry technique and amplification by biotinylated tyramide. The relationship between the expression of HIF-1α and the temporal evolution of ischemia has also been evaluated. As pathomorphological diagnosis of early myocardial ischemia has many problems in human autopsy material with less than 4 to 6 h after clinical onset, we suggest that HIF-1α is helpful in the early acute myocardial infarction diagnosis, so it stains necrotic areas within the first 2 h. The amplification procedure provides a higher intensity of the final staining without losing specificity. It is concluded that in normal cardiac fibers, basal expression of HIF-1α is not appreciable, but it steadily increases after ischemia. With regard to the practical applicability in forensic field, our observations suggest that positive immunohistochemical expression of HIF-1α on heart samples may be used as a reliable indicator of myocardial damage in cases without cardiac lesion evidence, using conventional microscopy. This method is especially useful and may provide definitive proof of myocardial ischemia in unexpected deaths without previous symptoms, or in forensic cases with a short period of clinical manifestations. In addition, it may have been involved in possible future cardiovascular therapies.

KEYWORDS: forensic science, forensic pathology, myocardial ischemia, heart, hypoxia-inducible factor-1 alpha, immunohistochemistry

Oxygen availability plays an important role in the regulation of expression of many different genes including erythropoietin (1–3), nitric oxide synthase (NOS) (4,5), glucose transporters (6,7), hypoxia-inducible factors (HIF), and vascular endothelial growth factors (VEGF) (8), that are necessary for the maintenance of the homeostasis in hypoxic conditions.

Regarding the molecular and biochemical mechanisms underlying cardiac responses to hypoxia and to ischemia/reperfusion injury, we have focused our attention on HIF-1.

The major transcription factor that is involved in the adaptive response to hypoxia is HIF-1. It consists of HIF-1 alpha (HIF-1α) and beta subunits (HIF-1β). HIF-1α has been identified as a bHLH-PAS family member, which is instrumental in the oxygen-dependent regulation of these genes (9,10). HIF-1α promotes neovascularization in response to myocardial ischemia by the activation of the transcription of the gene encoding vascular endothelial growth factor (8,11). It rapidly accumulates in nuclei upon exposure to hypoxic conditions where it heterodimerizes with aryl hydrocarbon nuclear receptor translocator, ARNT, also referred to as HIF-1β (12).

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In animal models, previous studies have demonstrated that the induction of HIF-1α protein occurs without a concomitant change in its mRNA level during the early responses due to two different mechanisms: ischemia and mechanical stress (13). According to this author, HIF-1α and VEGF are induced in the nonhypoxic myocardium, remote from the ischemic area, in hearts, which are subjected to mechanical stresses *ex vivo* and *in vivo*. In the human model, other authors have defined a molecular level at the sequential expression of HIF-1α and VEGF in the human heart during ischemia (14).

In the present investigation, we attempt to obtain quantitative and qualitative expression of antibody HIF-1α, in specimens of heart tissues that are affected by various degrees of ischemic insult with immunohistochemical method. We have also evaluated the relationships between the expression of HIF-1α and temporal evolution in human ischemic hearts.

Materials and Methods

We studied 48 human hearts; they were obtained from the medico legal autopsies performed at the Department of Forensic Medicine and Pathology of Santiago de Compostela, Ministry of Justice (Spain) and two cases of colorectal carcinoma from the Department of Pathology, Faculty of Medicine, Santiago de qCompostela (Spain). Cases with advanced autolysis or putrefaction were refused. In these selected cases, a complete medico legal autopsy was performed within 12 h, including toxicological analysis.

TABLE 1—*Details of populations included in study.*

Population	Subpopulation	Details and postmortem findings	Number of cases	Age range (years)	Observations
1		Macroscopic infarction	9	30–91	Two cases of colorectal adenocarcinoma were add as control of technique
2		Circumstances suggestive of sudden cardiac death but no macro nor microscopic evidence of myocardial ischemia	24	39–86	
	I	Duration of symptoms ≤ 2 h	9	56–82	
	II	Duration of symptoms >2 h	7	39–73	
	III	Without symptoms	8	47–86	
3		Miscellaneous deaths without evidence of myocardial ischemia	15	35–51	

For the discriminatory analysis, we chose the diagnostic category as the grouping variable, establishing three groups: cases with evidence of macroscopic myocardial infarction ($n = 9$) and colorectal adenocarcinoma ($n = 2$) were considered as positive controls (population 1); cases of sudden cardiac death with suggestive circumstances of antemortem myocardial ischemia but neither macro- nor microscopic evidence of ischemia (focal eosinophilia, hydropic swelling, loss of nuclei, coagulative necrosis, contraction band necrosis, areas with loss of cross-striation, waving and rupture of the cardiac myocytes) ($n = 24$), were considered as problem cases (population 2). In that group, three subpopulations were established with the following criterion: subpopulations I consist of cases with duration of previous symptoms ≤ 2 h ($n = 9$), subpopulations II consist of cases with duration of previous symptoms >2 h ($n = 7$) and subpopulations III consist of cases without previous symptoms ($n = 8$); noncardiac death, such as cranial gunshot wounds and falls from height ($n = 15$) without myocardial damage, were considered as negative controls (population 3).

Myocardial tissues were obtained from potential ischemic and nonischemic regions. In each case, nine samples were taken from the circumference of a transversal section of the heart halfway between the valvular plane and the apex, including the septum, anterior, lateral, and posterior walls of the left ventricle, and one more block from the lateral wall of the right ventricle. Tissue was fixed in 4% neutral-buffered formalin (phosphate buffer 0.1 M pH 7.4) for 18–24 h. Fixed tissues were then dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin. Tissues were sectioned to a thickness of 4 μm, mounted on slides, and stained with hematoxylin–eosin (H&E) and thrichrome of Masson.

In all cases, new sections of myocardial tissue were obtained for immunohistochemical study. Sections were placed on Histobond adhesion Microslides (Marienfeld, Landa-Königshofen, Germany). Slides were immunostained with pretreatment (heat-induced epitope retrieval). Epitope retrieval was performed by boiling the slides in monohydrated citrate buffer (pH 6.0, 0.01 M) in a water bath (100°C, 40 min). Endogenous peroxidase activity was blocked treating the sections with 0.3% H_2O_2 in phosphate-buffered saline for 30 min. For immunohistochemical technique, HIF-1α monoclonal antibody was used at dilution of 1:100 (Novus, Biologicals, Littleton, CO). The detection system employed was the catalyzed signal amplification (CSA) (DakoCytomation, Carpinteria, CA) following the instructions provided by the manufacturer. Counterstaining was performed with hematoxylin. All cases of this group were registered on an index card with the following information: case number, gender, age, cause of death, postmortem interval, toxicological screening, previous symptoms and duration, and finally HIF-1α staining pattern, including type of cellular structures marked as nuclear, cytoplasmic, and mixed or none.

Results

From the forensic samples (48 human hearts), 27 were males and 19 female. The average age was 50.6 years (range 30–91 years). As a whole, cases were classified as it is shown in Table 1. In population 1 (age range 30–91), due to brief survival time, eosinophilic infiltration was not seen in any case. In all the cases, cells also showed localized bleeding and dissociation. Erythrocytes were sometimes arranged in rows between myocardial cells. All the cases that were used as positive control, including two cases of colorectal adenocarcinoma, showed intense staining mixed pattern (nuclear and cytoplamic) by immunohistochemical method with HIF-1α. Positive staining was also observed in endothelial cells of small vessels, especially on colorectal adenocarcinoma.

Population 2 was composed of 24 cases (age range 39–86) with areas of apparently healthy muscle without visible abnormalities on H&E and Masson's trichrome staining. Positivity to HIF-1α antibody was seen in 79.1% ($n = 19$). Most of these part showed nuclear positivity, followed by staining mixed pattern (nuclear and cytoplasmic). Regarding subpopulations, in type I (symptoms ≤ 2 h), it was composed of nine cases (age range 56–82). In that subpopulation, seven cases have shown nuclear positivity, one case mixed, and one case none. Exclusive cytoplasmic positivity was not seen in this group. In subpopulation type II (symptoms >2 h), which was composed of seven cases (age range 39–73), one case has shown nuclear positivity, four cases have shown mixed positivity, and three cases with no positivity. Finally, in subpopulation III (without previous symptoms), which was composed of eight cases (age range 47–86), two of them had nuclear staining and four mixed. Exclusive cytoplasmic staining was not observed in this group. In such 19 cases with myocardial ischemia evidence (positivity to antibody), cause of death was established as myocardial infarction and fatal arrhythmia. In the remaining five cases, the cause of death was undetermined. Further information about demographic and immunohistochemical patterns of staining are provided in Table 2. Cases of population 3 ($n = 15$) showed no positive staining to HIF-1α.

Discussion

In adults, angiogenesis occurs in response to tissue hypoxia/ischemia and plays an important role in determining the progression of ischemic heart disease. A critical molecular pathway induced by hypoxia/ischemia is the activation of HIF-1. According to Jiang et al. (15) and Semenza (16,17), another argument in support of this point of view is that the expression of the gene for HIF-1α is exquisitely sensitive to cellular hypoxic conditions, making it one of the earliest effectors of the response to ischemia. How cells sense ischemic stimuli and how this translates into gene

TABLE 2—*Demographic characteristics and postmortem findings in population 2.*

Case no	Gender	Age	Postmortem interval (h)	Previous symptoms	Duration of symptoms	Coronary atherosclerosis (% of narrowing)*	Coronary thrombi	Toxicological screening	Staining pattern to HIF-1α
1	Male	59	6	No	—	I	No	Negative	None
2	Female	69	9	Neck and chest pain	4 h	II	No	Negative	Mixed
3	Male	62	9	Chest pain	2 h	III	No	Negative	Nuclear
4	Male	86	7	No	—	IV	No	Negative	None
5	Male	53	2	No	—	I	No	Negative	Nuclear
6	Male	39	6	Chest pain, vomits	9 h	II	Yes	Cocaine, alcohol	Mixed
7	Female	58	3	Neck pain, vomits	1 h	IV	No	Negative	Nuclear
8	Male	64	2	Chest pain	40 min	II	No	Negative	Nuclear
9	Female	80	4	No	—	II	No	Negative	Nuclear
10	Female	71	7	Chest pain, nauseas	30 min	IV	No	Benzodiacepine, alcohol	Nuclear
11	Male	65	5	Chest pain	4 h	I	No	Caffeine, alcohol	Mixed
12	Male	60	9	Neck and chest pain, nauseas	3 h	III	Yes	Negative	Nuclear
13	Male	80	6	Chest pain	50 min	II	No	Negative	Nuclear
14	Male	56	6	Vomits, neck pain	20 min	IV	No	Negative	None
15	Female	70	11	Abdominal and chest pain	2 h	IV	Yes	Negative	Nuclear
16	Male	82	2	Chest pain	2 h	IV	Yes	Negative	Mixed
17	Female	47	7	No	—	I	No	Cocaine	Mixed
18	Male	58	6	No	—	I	No	Alcohol	Nuclear
19	Male	49	10	No	—	IV	No	Negative	Mixed
20	Male	72	4	Chest pain, nauseas	1 h	I	No	Alcohol	Nuclear
21	Male	73	2	Abdominal pain, nauseas	3 h	III	Yes	Negative	None
22	Female	70	2	Abdominal and chest pain, nauseas	10 h	IV	No	Negative	Mixed
23	Female	65	9	Abdominal and chest pain, nauseas	8 h	IV	No	Alcohol	None
24	Female	69	6	No	—	II	No	Negative	Mixed

*Grade of coronary atherosclerosis: *Grade I: 0–25% of narrowing; Grade II: 25–50%; Grade III: 50–75%; Grade IV: >75%.*
HIF: 1α, hypoxia-inducycle factor 1 alpha.

expression, in our opinion, is not well understood, but recent evidences suggest that HIF-1α may exert both proapoptotic (7) and antiapoptotic actions in response to hypoxia in vascular endothelial cells stressed by anoxia (18).

With regard to methodology, preliminary results of immunohistochemical method without amplification of signal were poor and unsatisfactory. Therefore, we used the amplification procedure, as deposition of biotinylated tyramine (BT) resulted in a tremendous increase in sensitivity of antigens immunohistochemically, especially when gluteraldehyde-fixed tissues are processed as in our cases (19). With standard immunostaining protocol, it needs incubation for a long time in diaminobenzidine (DAB) to produce color reaction. After BT amplification, the DAB reaction occurs rapidly. This study shows that the biotin amplification produced has a great utility in immunohistochemical technique for HIF-1α antibody, because it provides greater sensitivity with reduced or without background staining.

According to observations of Kuwai et al. (20) and Jiang et al. (21), expression of HIF-1α was increased in tumor tissues compared to the corresponding normal mucosa. Due to those findings, we have extended the inmunohistochemical analysis with colorectal adenocarcinoma samples as quality control to our method, which showed a similar tendency with mixed staining pattern (Fig. 1). Extreme care should be used to identify and verify positive reactions, because cross-reactions (antibody gives positive reaction with other antigens different than the ones used to obtain it) and false positive are common in cases of putrefaction or delay in fixation (22). Such phenomenon commonly occurs by incomplete blocking of endogenous peroxidase or biotin's activity. While using monoclonal antibody like HIF-1α, cross-reactions and false positive are infrequent, but in order to avoid them, we reject all cases with minimal putrefaction or advanced autolysis. On the other hand, false negative often occurs due to prolonged fixation of tissue (22), thus that is why we made fixation for short time (<24 h).

Firstly, our study shows that all specimens with macroscopical evidence of acute myocardial infarction (positive controls, $n = 9$) showed intense immunoreactivity for HIF-1α. In these cases, a marked accumulation of HIF-1α was evident in both cytoplasm

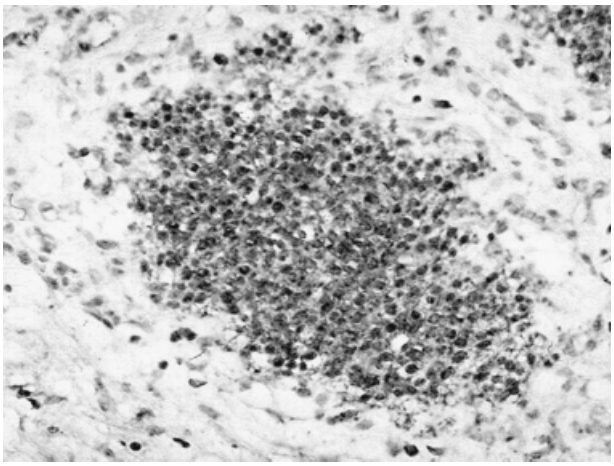

FIG. 1—*Strong mixed (nuclear and cytoplasmic) staining in colorectal adenocarcinoma used as control of the immunohistochemical method. Original magnification × 200.*

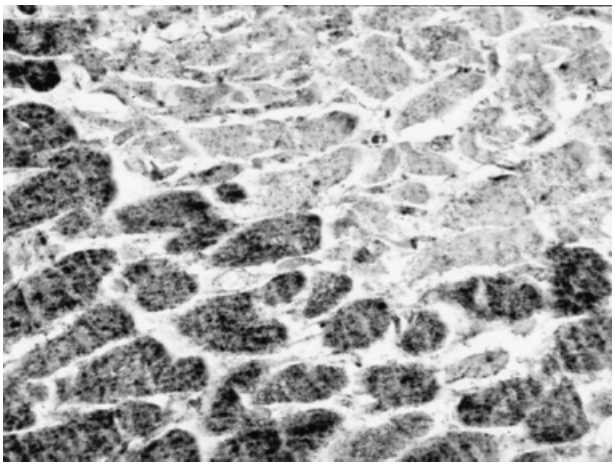

FIG. 2—*Homogeneous granular cytoplasmic and nuclear pattern staining in myocyte was shown in population 1 (macroscopic infarction). Original magnification × 400.*

FIG. 4—*Ischemic cardiomyocytes with hypoxia-inducible factor-1 alpha antibody in nuclei (white arrow) in contrast with hematoxylin-stained quiescent nuclei in noninfarcted cardiomyocytes (black arrows). Original magnification × 600.*

and nuclei of ischemic myocytes (Fig. 2). In most cases, cytoplasmic staining showed granular or vesiculated pattern. When myocardial infarction was well developed (older than 24 h), the nuclear localization of HIF-1α in the ischemic region could not be counted because the morphology of myocytes was too shrunk to identify the nuclei. On the other hand, expression of antibody in areas of collagen associated with old myocardial infarction was negative (Fig. 3).

We could not demonstrate the presence of immunostaining for this antibody in negative control cases (population 3). In consequence, immunostaining was not detectable within cytoplasm endothelial cells, neither in cytoplasm of non-ischemic fibers. In our opinion, sensitivity and specificity of immunohistochemical technique was acceptable.

In population 2, immunostaining with HIF-1α was heterogeneous. Whereas these normal tissue sections displayed absence of immunostaining, those located in the ischemic area showed strong homogeneous staining of myocytes and endothelial cells of small blood vessels. In cases with very early myocardial ischemia (symptoms), immunostaining was mostly nuclear. In the ischemic region, the increase in the HIF-1α expression was observed within 2 h after presentation of symptoms of ischemia and continued to increase the intensity according to temporal evolution. We checked that in ischemic progression, granular and vesiculated staining pattern became more homogeneous and diffuse pattern, with increase of signal intensity. In this group, immunopositivity for HIF-1α was seen in 19 of 24 cases scrutinized (79.1%), but none of these cases showed histological evidence of ischemia with conventional techniques (H&E and Masson's trichrome). We found three cases with symptoms (range 20 min–8 h) but no staining. We cannot offer explanation to this phenomenon; nevertheless, we think that symptoms of ischemia are not synonymous with irreversible myocardial damage. In group of negative cases, there was one case with short interval of ischemia (20 min), maybe not enough to develop cellular damage. The remaining two cases had longer intervals of ischemia (3 and 8 h). This behavior could be explained by sampling error to histological examination.

Regarding spatial distribution of staining, immunoreactivity was detected in both endothelial and myocardial cells in all specimens of human hearts that were affected by ischemia or infarction. Signal was concentrated primarily within nuclei of ischemic myocytes and endothelial cells lining the small blood vessels (Fig. 4). Nuclei of cells that had incorporated the HIF-1α stained deep brown as opposed to the hematoxylin-stained quiescent nuclei (Fig. 5). In the endothelium of medium-sized vessels, the level of expression of HIF-1α was less than the level in the ischemic myocardium. This observation suggests that the angiogenic effects of HIF-1α are limited to regions of terminal small vessels in the myocardial tissue.

Interestingly, we have observed in borderline areas of infarcts homogeneous immunostaining in cytoplasm, but nuclei were unstained (Fig. 6). In view of the known role of the HIF-1α, as transcriptional activator of genes encoding vascular endothelial growth factor and other important mediators of angiogenesis, we have considered the possibility that this phenomenon was due to HIF-1α contribution to the limitation of infarct size by promoting angiogenesis and vascular remodeling. In our opinion, these findings should be interpreted with caution, but suggest that HIF-1α exerts an important role in autolimitation of myocardial infarction.

We think the method is useful in sudden natural deaths or forensic field, because this technique might help to demonstrate

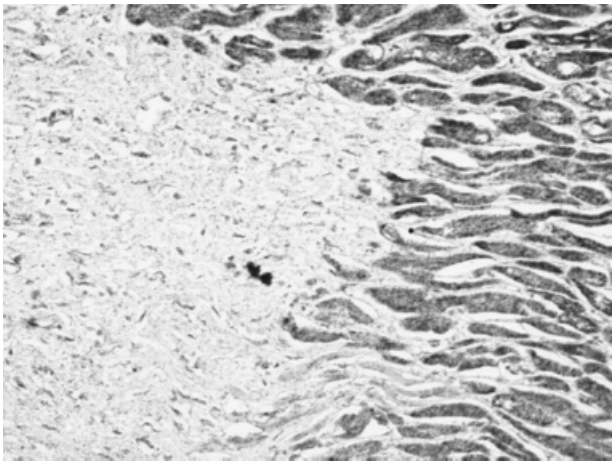

FIG. 3—*Negative immunostaining in old myocardial infarct (collagen scar), and positive staining in surrounding cardiac tissue. Original magnification × 200.*

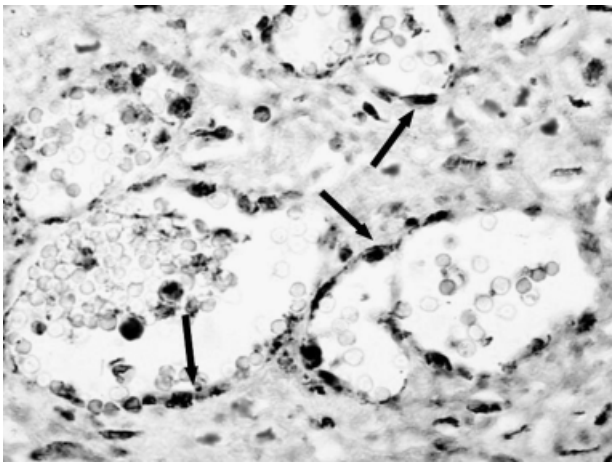

FIG. 5—*Endothelial blood vessel cells showing localization of hypoxia-inducible factor-1 alpha in the nuclei (arrows). Original magnification* × *400.*

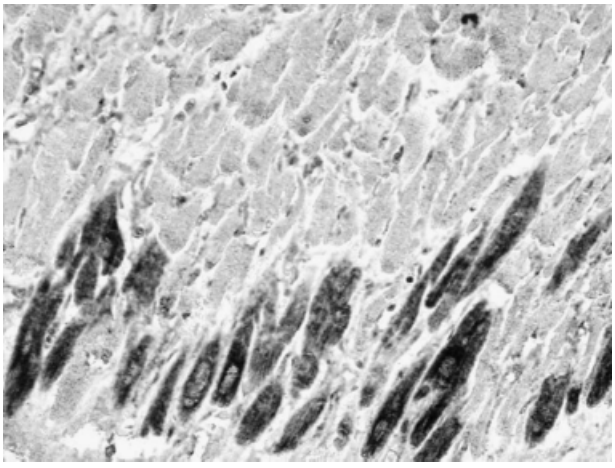

FIG. 6—*Borderline zone of myocardial infarct showing positive cytoplasmic staining and nuclei unstained. Original magnification* × *400.*

early myocardial ischemia, but it is also useful in selected cases or situations in which a person is involved in a motor vehicle collision and the question arises as to whether or not underlying natural disease may have caused the individual to have collision.

In the clinical setting of myocardial infarction, it is likely that damaged myocardial cells also release HIF-1 (both subunits) molecules. Thus, in relation to cardiovascular treatments, previous studies have reported that pharmacologic manipulation of HIF-1 levels may provide a novel therapeutic to myocardial infarction (23,24).

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